MICRO-ANALYSIS IN MEDICAL BIOCHEMISTRY

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BY

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used as a general instrument for all colorimetric methods of analysis.

In order to make room for new procedures, without unduly increasing the size of the book, some material of the first edition has been omitted, e.g., the artificial standards and methods which were developed during the war for the easy estimation of blood, C.S.F. and urine constituents in field laboratories.

It has always been intended that this should be a book to use, rather than a text-book to read; and all statements and descriptions have been kept to the minimum length compatible with clarity of instruction. For this reason the brief statements of the use that may be made of each determination in the investigation of disease may appear cryptic and inadequate to the clinician. But the few criticisms which have reached me on this score are greatly outweighed by the approval of the many laboratory workers, clinical pathologists and students, who have found these brief reminders of biochemical abnormalities a ready and easy means of refreshing their memories on matters about which they have previously read extensively in text-books of clinical medicine, but have in part forgotten.

To my colleagues and friends, who have generously contributed of their time and thought to the testing and selecting of new methods and the checking of modified procedures, my sincere thanks are due. In this regard I am especially grateful to Prof. R. H. A. Plimmer, Dr. I. D. P. Wootton, Dr. W. Klyne, Dr. G. M. Bull, Dr. D. A. K. Black and Dr. S. P. V. Sherlock; and to Miss V. Bentley, Miss M. Cox, Dr. R. M. Haslam and Miss V. R. Pash for their careful checking of proofs and preparation of the index.

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MICRO-ANALYSIS IN MEDICAL BIOCHEMISTRY

Chapter I

NORMAL VALUES

By 'normal value' is meant the amount of a constituent sent in the body fluid or excretion of a healthy human g. In fact, this amount varies over a range; and, while t healthy persons can be included in a class having the pted 'normal' amount, some individuals are found to v divergent figures. Such exceptional individuals are often entirely 'normal' in all other investigated respects. The judgment, therefore, of whether a given analytical figure is 'normal' will depend on the experience and total data at the command of the interpreter of the result.

The values given in Table 1 are taken from the literature and from our accumulated results. In most cases they apply to the method of analysis given here. In others they are the result of a direct comparison between the method given and a method which has been 'standardized for normal human beings.' Blood values are for fasting persons.

The figures for whole blood, plasma and serum are based on a survey by Wootton, Maclean Smith and King (1950) of about 80 normal adults aged 20 to 50 years. It would have been convenient to express results as an average normal figure with a standard deviation. The plotted results for blood values have usually given a 'skew' type of distribution curve, however; and they have been listed, therefore, in terms of a range. 80 per cent of the normal values given fall between the upper and lower 10 per cent limits, and 98 per cent between the upper and lower 1 per cent limits. Thus 1 per cent of normal subjects have blood urea values lower than

TABLE 1

Normal Values

	mg. per 100 ml.*			
	Lower 1% level	Lower 10% level	Upper 10% level	Upper 1% level
WHOLE BLOOD				
Urea	12	16	35	47
Non-protein nitrogen	25	29	43	51
Urie Aeid	0.6	1.6	3.9	4.9
Creatinine	0.1	0.1	1.2	2.6
Phosphate, inorganic (as P)—				
Adult		2	3	
Child		4	5	
Cholesterol	115	140	215	265
Sugar	55	68	96	109
Chloride (as NaCl)		450	510	
PLASMA				
Total Protein	6.3	6.7	7.7	8.2 g.
Albumin	4.0	4.4	5.3	5.7 g.
Globulin	1.5	1.9	2.8	3.0 g.
(Ratio alb./glob. = 1.3–3.6)				
Fibrin		0.2	0.4	g.
Bilirubin	0.1	0.1	0.5	0.8
Chloride (as NaCl)	581	593	620	632
Sodium (as Na)	306	316	340	350
CO_2-combining power		56	74 vol.	
Phosphate (as P)—				
ester		1	2	
inorganie		2	3	
lipid		7	10	
Phosphatase, Acid	0.8	1.2	3.1	4.6 units
Acid, formol stable	0	0	2.1	4.1 units
Alkaline	3.3	4.5	9.5	12.9 units
Amylase	71	91	163	209 units
SERUM				
Calcium	9.1	9.6	10.9	11.4
Sodium	307	318	342	353
Potassium	13.5	15.1	19.6	21.7

CEREBRO-SPINAL FLUID	mg. per 100 ml.
Protein	20–40
Globulin (Pandy and Nonne Apelt Test)	absent
Chloride (as NaCl)	700–740
Sugar	60–100
Urea	15–30
Calcium	4–5
Creatinine	0.7–1.5
CO_2-combining power	55–65 vols.

FÆCES	Percentage by weight of dried fæces
A. Total fat	15–25
B. Unsoaped fat = (Neutral fat + Free fatty acid)	10–15
C. Free fatty acid	9–13
D. = A − B = Fatty acid present as soap	10–15
E. = B − C = Neutral fat	1–2

*Except where stated otherwise.

12 mg., 9 per cent between 12 and 16 mg., 80 per cent between 16 and 35 mg., 9 per cent between 35 and 47 mg., and 1 per cent over 47 mg. per 100 ml. In clinical practice any single

TABLE 2

Abnormalities in Composition of Human Blood

Constituent	Clinical conditions in which high values (unless otherwise stated) are found
Amylase	Acute pancreatitis.
Bilirubin	Jaundice.
Calcium (serum)	Hyperparathyroidism. Low in tetany (infantile), parathyroidectomy, severe nephritis, cœliac disease.
Chlorides (whole blood)	Nephritis, some cardiac conditions, eclampsia, prostatic obstruction, anæmia.
Chlorides (plasma)	Low in pneumonia, fever, diabetes ; all cases of dehydration, such as gastro-intestinal disturbances associated with diarrhœa and vomiting.
Cholesterol	Biliary obstruction, nephritis, nephrosis, diabetes, pregnancy. Low in pernicious anæmia.
CO_2-combining power	Alkalosis ($NaHCO_3$ administration, intestinal obstruction). Low in acidosis (diabetes, starvation, and severe nephritis).
Creatinine	Nephritis—only in severe cases above 4mg. per 100 ml.
Non-protein N	Nephritis, eclampsia, intestinal obstruction, etc.
Phosphatase (alkaline)	Generalized bone disease, obstructive jaundice.
Phosphatase (acid)	Carcinoma of the prostate.
Phosphates	Nephritis. Low in rickets.
Plasma proteins (total)	Anhydremia. Low in nephritis with œdema (nephrosis), starvation.
Plasma albumin	Low in nephrosis.
Plasma globulin	Nephrosis, anaphylactic conditions, hepatitis.
Potassium	Addison's disease, terminal nephritis.
Sodium	Low in Addison's disease.
Sugar	Diabetes, hyperthyroidism.
Urea	Nephritis, intestinal obstruction, cardiac failure, etc.
Uric Acid	Nephritis, eclampsia, arthritis, gout.

result falling *outside* the 10 per cent limits is considered suspicious ; a result which is *outside* the 1 per cent limit is almost certainly abnormal.

Milli-equivalent values per litre of blood are listed for many constituents in the individual sections for each substance.

In studies of acid-base balance it is often desirable to express the concentrations of those constituents of plasma

TABLE 3

Approximate Average Daily Composition of Human Urine

	mg. per 100 ml.	g. per 24 hr.	As nitrogen g. per 24 hr.	Milli-equivalents per 24 hr.
NITROGENOUS CONSTITUENTS				
Urea	2000	30	14	
Ammonia	50	0.8	0.7	50
Creatinine	100	1.5	0.56	
Hippuric Acid	30	0.5	0.04	3
Amino-Acids	400	6.25	1	
Uric Acid	25	0.4	0.17	2
Urochrome and other pigments				
SULPHUR-CONTAINING CONSTITUENTS				
Inorganic sulphates (as H_2SO_4)	120	1.8		36
Ethereal sulphates, e.g. indican	20	0.3		2
'Neutral' sulphur compounds, e.g. NaCNS	20	0.3		
OTHER CONSTITUENTS				
Organic				
Oxalic acid	1	0.02		0.5
Carbonic Acid				
Aromatic hydroxyacids, e.g. *p*-hydroxyphenylacetic				
Inorganic				
Phosphate (as P)	110	1.7		100
Chloride (as NaCl)	800	12		
Chloride (as Cl)	485	7.3		200
Sodium	670	10		430
Potassium	170	2.5		65
Calcium	13	0.2		10
Magnesium	13	0.2		16
Water		1500		

which act as acids or bases in terms of equivalents, so that the 'total acid' of the plasma may be compared with the 'total base,' thereby demonstrating if there is a preponderance of acid over base, or *vice versa*, i.e. whether the 'acid-base

balance' favours an acid or an alkaline state. It may be convenient, therefore, to express the results for the acid radicles [chloride, carbon dioxide (bicarbonate), phosphate, sulphate, proteins and organic acids] and the basic (sodium, potassium, calcium, magnesium) as milli-equivalents per litre of plasma ; the equivalent, that is, of the amount of acid or base which they represent, or are capable of neutralizing. The plasma proteins, for instance, act as weak acids, and neutralize a certain quantity of base ; and it is possible to express their concentration, as one would for an acid, in terms of the amount of the base they neutralize. This is done by dividing the concentration of protein (in mg. per litre) by the average equivalent weight of the plasma proteins, i.e. by that fraction of their molecular weight which represents the amount which would neutralize one milli-equivalent of sodium hydroxide (i.e. 1 litre of N/1000 NaOH). This is most easily done by the use of a factor : g. protein (per 100 ml.) $\times 2.43$ =milli-equivalents per litre. With simple monovalent ions like sodium and chloride the mg. per litre are divided by the atomic weight ; with divalent ions (e.g. calcium) by half the atomic weight.

Chapter II

PROCEDURES FOR WHOLE BLOOD

TAKING OF CAPILLARY BLOOD

Blood may be taken from a puncture in the ear or finger, but the most convenient place to obtain capillary blood is probably from the thumb over the bed of the nail. The part is wiped clean with a little ether or spirit and a stab of 1 to 2 mm. deep is made by means of a puncturing apparatus, Hagedorn needle or sharp fragment of glass. A piece of soft rubber tubing or of gauze is wrapped fairly tightly about the thumb above the knuckle. On flexing the thumb a free flow of blood is usually obtained. If the blood does not come easily, the rubber is released and the hand shaken in a downwards direction. This operation will ensure an adequate amount of blood when the tourniquet is replaced and the thumb flexed. The pipette is held horizontally with its point in the drop of blood issuing from the stab wound. The blood is allowed to run in exactly to the 0.2 ml. mark. The pipette is then wiped and the blood allowed to run into a 15 ml. conical centrifuge tube containing water or isotonic sodium sulphate solution, and by alternate blowing and sucking the pipette is washed several times with the solution.

TAKING OF VENOUS BLOOD

When several different estimations on whole blood are required it may be preferable to take a venous sample. 2 ml. will usually suffice. The blood is withdrawn by a syringe from a vein in the antecubital fossa, according to the instructions given under *Plasma* (p. 37), and is placed in a tube or screw-cap bottle containing a trace of potassium oxalate (the residue from a micro-drop of 30 per cent dried in the tube at about 100° C.) or 1 drop of heparin (1000 units per ml.).

DEPROTEINIZATION OF BLOOD

Urea, sugar and several other substances are present in both the plasma and cells of blood ; but they diffuse easily out of the cells when the blood is diluted. Since there are definite advantages, for several analytical purposes, in leaving the cells intact so that interfering substances contained in them will not be liberated and appear in the protein-free extract of the blood, the custom of diluting the blood with an isotonic liquid, rather than laking it with water, has been adopted (Herbert and Bourne, 1930). Isotonic sodium sulphate solution is used, rather than sodium chloride, because the sulphate ion interferes less in the chemical reactions used than chloride does.

Many deproteinizing agents have been described. Those used here have been selected to fit the individual case ; and always for some special reason. Thus, copper tungstate is employed in the blood-sugar method because it is the most efficient means of eliminating the small amount of non-sugar reducing substances in the plasma, i.e. in the unlaked diluted blood. But it cannot be used for the colorimetric urea method, because of interference with Nessler's reagent ; nor in the non-protein nitrogen procedure, since it precipitates some of the non-protein nitrogen-containing substances. Similarly, the zinc hydroxide deproteinization used for urea is useless for sugar methods which are based on alkaline copper reagents, because of a depression of the activity of the copper reagent by small amounts of zinc which are present in the filtrate (King, Haslewood and Delory, 1937*b*). Nor should it be used for creatinine or phosphate, since it precipitates part of these constituents, and low results would be obtained. For substances like phosphate and sulphonamides it is necessary to use a fairly strongly acid protein-precipitating agent, in order to extract them completely ; or to keep them in an analysable condition as with ascorbic acid.

UREA

Urea represents about 50 per cent of the non-protein nitrogen of the blood. Normally there are between 16 and

35 mg. of urea present per 100 ml. High values are found in conditions associated with impaired renal function—particularly in chronic nephritis, but also in some cases of acute nephritis, cardiac failure, prostatic obstruction, intestinal obstruction, etc.

PRINCIPLE

The sample of blood is digested with urease, and the urea thus converted into ammonia. After the removal of proteins, the colour produced by the ammonia with Nessler's reagent is compared colorimetrically with the colour produced under the same conditions with a standard ammonium chloride solution or with a standard urea solution treated with urease.

Direct Nesslerization should not lead to the production of cloudiness in the case of protein-free filtrates from unlaked blood. The sulphydryl substances, glutathione and ergothioneine, which produce turbidities with Nessler's reagent because of the insolubility of their mercury salts, are confined to the cells and do not appear in the filtrate, as is the case with filtrates of laked blood. Filtrates of unlaked blood have the further advantage that no ammonia is contributed to the determination through the action of the arginase of the red cells on the arginine contained in most commercial preparations of urease (see Addis, 1928). The use of zinc hydroxide as deproteinizing reagent eliminates a small amount of turbidity-producing substance contributed by most preparations of urease.

METHOD

Test. 0.2 ml. of blood (or of plasma) is added to a centrifuge tube containing 3.2 ml. of isotonic sodium sulphate solution. (0.2 ml. of standard urea solution, similarly treated, is a useful check on the method, including the activity of the urease.)

A 'knife-point' (about 20 mg.), or small 'spoon' (5 mm. of glass tube, 3 mm. internal diam., fused at right angles on the end of a glass rod), of Jack Bean* meal is added, and the tube

* The 'Arlco' Jack Bean meal (Arlington Chemical Company, Yonkers, New York) is a very suitable preparation. Others have appeared to be not as potent in urease activity, or as good in keeping quality. A crushed "urease tablet" may be similarly used. (N.B. The use of too much urease may lead to clouding when the Nessler reagent is added.)

stoppered with a rubber bung, mixed, and incubated at 37°C. for 20 minutes. 0.3 ml. of zinc sulphate solution and 0.3 ml. of 0.5 N-sodium hydroxide are added to precipitate the proteins. The mixture is *well* mixed by inversion after each addition and is then centrifuged, or filtered through a small (7 or 5.5 cm.) paper. 2 ml. of the supernatant fluid (representing 0.1 ml. of blood) are treated with 5 ml. of ammonia-free distilled water and 1 ml. of Nessler's reagent.

Standard. The solution is compared in a colorimeter with a 'low' or 'high' standard made up with 2 ml. and 5 ml. of the standard ammonium chloride solution (0.01 mg. of nitrogen per ml.), 5 ml. and 2 ml. respectively of water, and 1 ml. of Nessler's reagent.

Blank. For photoelectric measurement of the colour it is advisable to make the zero setting of the instrument with the solvent used in the estimation, usually water. A 'reagent blank,' consisting of 7 ml. of water and 1 ml. of Nessler, should be read at the same time as the standard and test, and its reading subtracted from those of test and standard. Alternatively, the zero setting may be made with the 'blank' when it will not be necessary to subtract from the readings of test and standard.

The colorimetric comparison is made with the use of an Ilford blue (622) or a violet light filter (621, see p. 203).*

CALCULATION

Photoelectric Colorimeter.

(1) 'Low' standard :—

$$\text{Blood urea}\dagger \begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.02 \times \dfrac{100}{0.1} \times 2.14 \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 42.8 \end{cases}$$

* If the test reads more than double the standard, the colorimetric comparison should be repeated with 0.5 ml. of supernatant (≡0.025 ml. blood) *plus* 6.5 ml. of water ; (or 2 ml. of test diluted immediately with 6 ml. of blank).

† mg. per 100 ml. blood.

(2) 'High' standard :—

$$\text{Blood urea}^* \begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.05 \times \dfrac{100}{0.1} \times 2.14 \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 107 \end{cases}$$

N.B.—1 mg. of nitrogen≡2.14 mg. of urea

* mg. per 100 ml. blood.

Duboscq Colorimeter. The calculation is the same as the above, except that the ratio of readings is inverted to Reading of standard/Reading of test, since the colour is inversely proportional to the concentration with the Duboscq type of colorimeter, instead of directly proportional as with the photo-electric. The 'blank' is not used.

SOLUTIONS

Nessler's Reagent. 11.3 g. of iodine crystals are weighed on a rough balance and dissolved in a solution of 15 g. of potassium iodide in 10 ml. of water. To 15 g. of mercury in a glass-stoppered reagent bottle is added most of this solution, and the mixture, kept cool in water, is shaken until the supernatant liquid has lost its yellow colour. This supernatant liquid is then decanted into a 100 ml. flask and a drop tested with 1 per cent starch. If no colour is obtained, more of the iodine solution is added until a drop of the mixture gives a faint reaction with starch.

The total solution is then diluted to 100 ml. and poured into 485 ml. of 10 per cent sodium hydroxide. The solution, if turbid, should be filtered or allowed to settle before use, and should be kept in a bottle with a rubber stopper.

Standard Ammonium Chloride Solution (containing 0.01 mg. of nitrogen per ml.). 153 mg. of pure ammonium chloride (dried in a desiccator) are dissolved in water and the volume made to 100 ml. 25 ml. of this solution with 10 ml. of N-sulphuric acid are diluted to 1 litre with distilled water.

Standard Urea Solution. 100 mg. in 100 ml. water, preserved with a drop of chloroform and kept in the cold.

Isotonic Sodium Sulphate. 30 g. of crystalline sodium sulphate ($Na_2SO_4.10H_2O$), or 13.2 g. anhydrous Na_2SO_4, are dissolved in water and made to 1 litre.

Zinc Sulphate. 10 g. of crystalline zinc sulphate ($ZnSO_4.7H_2O$) are dissolved in water and made to 100 ml.

0.5 N-*Sodium Hydroxide.* This should be accurately prepared (see p. 187), and should be titrated against the zinc sulphate. 10.8–11.2 ml. should be necessary to produce a permanent pink colour with phenolphthalein, when titrated into 10 ml. of zinc sulphate diluted with water. It is best kept in a wax bottle.

NON-PROTEIN NITROGEN

The non-protein nitrogen containing substances of blood are urea (10–20 mg. N), uric acid (1–2 mg. N), creatinine (0.5–1 mg. N), amino-acid nitrogen (6–8 mg.), and substances such as glutathione and ergothioniene (5–10 mg. N per 100 ml. of blood). The normal range of non-protein nitrogen (N.P.N.) is from 29–43 mg. per 100 ml. Increased values are found in the conditions showing a high blood urea.

PRINCIPLE

The proteins of laked blood or plasma are precipitated by trichloroacetic acid. Part of the filtrate is digested with sulphuric acid until all the nitrogen is converted into ammonium sulphate. The ammonium salt is estimated colorimetrically with Nessler's solution, excess of which is used for the test in order to neutralize the sulphuric acid and give an alkaline medium.

METHOD

Test. 0.2 ml. of blood is laked with 3.2 ml. of water, or 0.2 ml. of blood in 3.2 ml. of isotonic sodium sulphate is laked by the addition of a drop of 1 per cent saponin, followed by vigorous shaking. 0.6 ml. of 25 per cent trichloroacetic acid is added and the mixture shaken and centrifuged or filtered through a small (5.5 or 7 cm.) paper.

1 ml. of the filtrate (≡0.05 ml. of blood) is evaporated in a test-tube with 0.2 ml. of 50 per cent sulphuric acid containing 1 per cent SeO_2 until the liquid turns dark and white acid fumes are evolved. (See p. 42 regarding the method of heating.) Heating is continued until the mixture is colourless and for 3 or 4 minutes more. To the cooled solution are now added 5 ml. of water and, after thorough mixing, 3 ml. of Nessler's solution.

Standard. The colour produced is compared in the colorimeter with the 'low' or 'high' standard used in the determination of blood urea, with the blue (622) or violet filter (621), and with the 'blank' in a photoelectric colorimeter (see p. 203).

CALCULATION

Photoelectric Colorimeter.

(1) 'Low' standard :—

$$\text{N.P.N.*} \begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.02 \times \dfrac{100}{0.05} \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 40 \end{cases}$$

(2) 'High' standard :—

$$\text{N.P.N.*} \begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.05 \times \dfrac{100}{0.05} \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 100 \end{cases}$$

* mg. per 100 ml. of blood or plasma.

Duboscq Colorimeter. The calculation is the same as above, except that the ratio of readings is inverted to 'Reading of standard /Reading of test' (see p. 196).

SOLUTIONS

Nessler's Reagent and *Ammonium Chloride* standard as described for urea.

50 *per cent Sulphuric Acid containing Selenium Oxide.* 50 ml. concentrated acid are allowed to run slowly and with shaking into 40 ml. of distilled water in a 100 ml. volumetric flask. Selenium oxide (1 g. SeO_2) is added and shaken until dissolved. The mixture is cooled to room temperature, made to the mark and mixed.

25 *per cent Trichloroacetic Acid.* 25 g. dissolved in water and made to 100 ml.

URIC ACID

Uric acid is normally present to the extent of 1.6–4 mg. per 100 ml. of blood. In gout, eclampsia and arthritis, and in certain conditions of renal impairment high values are found.

PRINCIPLE

Blood in isotonic sodium sulphate solution is treated with tungstic acid reagent. This precipitates the proteins, and addition of phosphotungstic acid reagent and sodium cyanide to the supernatant liquid produces a blue colour with the uric acid present. The colour is compared with that given by a standard solution of uric acid. The blood must not be laked, as interfering substances, such as glutathione and ergothioniene, would be liberated from the cells.

METHOD

Test. 0.2 ml. of blood or plasma is pipetted into 3.2 ml. of isotonic sodium sulphate in a 15 ml. centrifuge tube. 0.3 ml. of 10 per cent sodium tungstate and 0.3 ml. of 2/3 N-sulphuric acid are added. The tube is stoppered, its contents mixed gently by inversion, and filtered or centrifuged. 2 ml. of the supernatant liquid (≡0.1 ml. of blood) are treated with 0.3 ml. of Folin's uric acid reagent and 1 ml. of sodium cyanide-urea reagent.

Standard. At the same time a mixture of 1 ml. of the uric acid 'blood' standard (≡0.004 mg. uric acid), 1 ml. of distilled water, and 0.3 ml. of Folin's reagent is also treated with 1 ml. of sodium cyanide-urea reagent.

Blank. 2 ml. of water, 0.3 ml. of Folin's reagent, and 1 ml. of cyanide-urea.

The 3 tubes are placed in a boiling water-bath for 5 minutes, cooled, water added to 8 ml., and the solutions compared colorimetrically [red (608) or orange (607) light filter]; and with a 'blank' in a photoelectric colorimeter (see p. 203).

CALCULATION

Photoelectric Colorimeter.

$$\text{Uric acid}^* \begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.004 \times \dfrac{100}{0.1} \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 4 \end{cases}$$

* mg. per 100 ml. blood.

Duboscq Colorimeter. The calculation is the same as above, except that the ratio of readings is inverted to 'Reading of standard/Reading of test' (see p. 196).

SOLUTIONS

Isotonic Sodium Sulphate as under blood urea (see p. 10).

10 *per cent Sodium Tungstate.* 10 g. $Na_2WO_4.2H_2O$ dissolved in water and made to 100 ml.

2/3 N-*Sulphuric Acid.* 18 ml. concentrated H_2SO_4 added slowly to about 900 ml. water in a litre volumetric flask, shaken, cooled, made to the mark and mixed (see p. 186).

Sodium Cyanide-urea Reagent. 5 g. of sodium cyanide and 20 g. of urea are dissolved in water, and the volume made to 100 ml. The urea prevents clouding during the determination. This solution should be renewed about once a month.

Folin's (1934) *Uric Acid Reagent.*

1. Preparation of molybdate-free sodium tungstate :* a solution of 250 g. of sodium tungstate in 500 ml. of water is treated with N-hydrochloric acid until neutral to litmus paper. The solution is saturated with hydrogen sulphide, and allowed to stand 24 hours. It is then treated with 400 ml. of absolute alcohol, added gradually with constant shaking. The mixture, after standing for a further 24 hours, is filtered, and the precipitate washed with 50 per cent alcohol and dissolved in 375 ml. of water. 0.5 ml. of bromine is added, and the mixture boiled gently until the excess bromine is dispelled. Sodium hydroxide solution (40 g. per 100 ml.) is now added to the hot solution until the latter is alkaline to phenolphthalein. The cooled solution, filtered if necessary, is treated with 200 ml. of absolute alcohol, and allowed to stand for 24 hours. The white crystals are filtered off and dried in a desiccator.
2. Preparation of reagent : 100 g. of molybdate-free sodium tungstate are treated gradually with a solution of 30 ml. of 'syrupy' phosphoric acid (89 per cent) in 150 ml. of water. The mixture is boiled gently under reflux for 1 hour, decolorized as above with a drop of bromine, cooled and diluted to 500 ml.

Stock Uric Acid Standard (*Folin*) (1 mg. per ml.). 1 g. of uric acid is placed in a 1-litre flask. 0.6 g. of lithium carbonate is dissolved in 150 ml. of cold water. The carbonate solution,

* Some Analar specimens of sodium tungstate have been sufficiently pure for this purification to be unnecessary.

filtered if necessary, and warmed to 60°C., is added to the flask containing the uric acid, which is warmed under the hot tap. The warm mixture is shaken for 5 minutes, cooled at once under the tap, and treated with 20 ml. of formalin (40 per cent solution of formaldehyde) and enough water to fill half the flask. A few drops of methyl orange are added, and then, gradually with shaking, 25 ml. of N-sulphuric acid. The solution should turn pink when 2–3 ml. of acid remain to be added. The mixture is now diluted to 1 litre, mixed and stored in the dark in a stoppered bottle, when it will keep almost indefinitely.

Uric Acid 'Blood' Standard (0.004 mg. per ml.). 2 ml. of the above 'stock' standard solution are diluted with water and 1 ml. of 40 per cent formalin to 500 ml. This solution should be made up fortnightly.

CREATININE

PRINCIPLE

Creatinine gives a red colour with alkaline solutions of picric acid (Jaffe's reaction). A similar colour is also given by blood (and plasma) filtrates. It is not certain that the colour in this case is due to creatinine, but the substance which may thus be estimated as blood 'creatinine' is of some clinical importance. Calculated as 'creatinine' the normal values are 0.1–1.2 mg. per 100 ml. of blood. In advanced renal failure enhanced values may be found.

METHOD

Test. 0.5 ml. of blood is added to 4.5 ml. of 5 per cent trichloroacetic acid, and the tube is stoppered and shaken. The mixture is centrifuged, and 4 ml. of the clear supernatant fluid (≡0.4 ml. of blood) is neutralized with 0.5 ml. of 2 N-sodium hydroxide and used as 'test' solution.

Standard. For normal blood a standard is made by diluting 1 ml. of the creatinine 'blood' standard described below with 3.5 ml. of water.

Blank. 4.5 ml. of water.

The 'test,' standard and 'blank' are then treated at the same time with 0.5 ml. of freshly made alkaline picrate solution (see below). After not more than 15 minutes the solutions are read in the colorimeter, using a blue-green

light filter, such as Ilford's 623, or, better, the mercury green filter 807 (see p. 203).

CALCULATION

Photoelectric Colorimeter.

(1) 'Normal' standard :—

$$\text{Blood 'creatinine'}^{*}\begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.004 \times \dfrac{100}{0.4} \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 1.0 \end{cases}$$

* mg. per 100 ml. blood.

In cases where a very high blood 'creatinine' is found or expected, stronger 'standards' may be made by using larger quantities of the 'blood standard,' e.g. 2 or 4 ml. ; and by diluting 2 ml. of blood filtrate with 2 ml. of water for the test.

(2) 'Abnormal' standard (e.g. 4 ml. of 'blood standard' with 2 ml. of filtrate).

$$\text{Blood 'creatinine'}\begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.016 \times \dfrac{100}{0.2} \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 8 \end{cases}$$

Photometric Estimation for Creatinine

Duboscq Colorimeter. The amount of creatinine in blood is so small that the amount of colour in a test, which is contributed by the excess of reagent (alkaline picrate solution), is appreciable compared with that produced by the reaction of 'creatinine' with reagent. The difficulty of obtaining an accurate assessment of the colour due to creatinine can be overcome by using the colorimeter as a photometer in the manner described by Delory and Jacklin (1942).

The neutral grey screen (0.2 or 0.5D ; cf. p. 197) is placed on the left-hand rack of the colorimeter, and the Ilford (623) blue-green filter placed over the eyepiece. The standard solution is placed in the right-hand cup and its depth is adjusted until the two fields appear equal. The reading S is recorded. The test solution is now substituted in the cup to give the reading T. Finally, a blank is read (reading B).

CALCULATION

$$\text{Blood 'creatinine'}^{*} \begin{cases} = \dfrac{1/T - 1/B}{1/S - 1/B} \times 0.004 \times \dfrac{100}{0.4} \\ = \dfrac{\dfrac{B-T}{BT}}{\dfrac{B-S}{BS}} \times 1 \\ = \dfrac{S}{T} \times \dfrac{B-T}{B-S} \times 1 \end{cases}$$

* mg. per 100 ml. of blood.

If it is desired to avoid this calculation, a curve is made from the readings obtained with varying strengths of standard. Results can then be read off from this curve. Since different preparations of picric acid may contain varying amounts of impurities (e.g. dinitrophenol), it is advisable to check the curve for each new batch of picric acid.

SOLUTIONS

Trichloroacetic Acid. 5 g. dissolved in water and made to 100 ml.

2N-*Sodium Hydroxide.* See p. 187.

Creatinine 'Stock' Standard (containing 1 mg. of creatinine per ml.). 1.602 g. of pure creatinine zinc chloride are dissolved in N/10 hydrochloric acid solution and the volume made with the N/10 acid to 1 litre.

Creatinine 'Normal Blood' Standard (0.004 mg. per ml.). 4 ml. of the above 'stock' standard are treated with 10 ml. of N/10 hydrochloric acid and the volume made with water to 1 litre.

Alkaline Picrate Solution. Five parts (by volume) of a saturated aqueous solution of *pure* picric acid, containing about 15 g. picric acid per litre, are mixed fresh daily with 1 part of 10 per cent sodium hydroxide.

NOTE. The picric acid may be purified by recrystallization from glacial acetic acid.† It must be of such purity that when

† *Purification of Picric Acid.* An alternative method of purification is as follows : 200 g. of commercial picric acid are placed in a litre beaker (or flask). 350 ml. of 10 per cent sodium hydroxide are added and the mixture heated on a boiling water bath, with stirring, until all the picric acid has dissolved ; extra hot water being added, if necessary, until solution is complete. To the hot, red solution of sodium picrate are added slowly with stirring 70 g. of sodium chloride. The mixture is cooled to 30°C. with occasional stirring. The precipitated sodium picrate is filtered on a Buchner funnel, washed with 5 per cent sodium chloride solution and sucked dry. The picrate is transferred to a

10 ml. of a saturated aqueous solution are treated with 5 ml. of N-sodium hydroxide, the colour (determined in the colorimeter) of the alkaline mixture so formed is not more than twice as deep as that of the saturated picric acid solution. The alkaline picrate must be prepared fresh daily for use.

INORGANIC PHOSPHATE

The blood of normal adult persons contains 2–3 mg. per 100 ml. of inorganic phosphate (expressed as P). In conditions involving an acidosis, such as is often found in nephritis, the amount present may be definitely raised. The amount of phosphate present in the blood of children, where bone formation is not yet complete, is at a higher level—usually of about 5 mg. per 100 ml. In rachitic conditions the figure is lowered.

PRINCIPLE

The inorganic phosphate of a deproteinized filtrate of the blood is coupled with molybdate, and the yellow phosphomolybdate is reduced with stannous chloride to give a blue substance. The amount of blue colour produced in the solution is directly proportional to the amount of phosphate present. (For the differential estimation of the different fractions of phosphate, see p. 63.)

METHOD

0.1 ml. of whole blood or plasma is pipetted into 4.9 ml. of 5 per cent trichloroacetic acid. The mixture is shaken well, and filtered after 5 minutes.

Test. 2 ml. of the clear filtrate (≡0.04 of blood or plasma).

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similar beaker which is half-filled with boiling water and stirred until dissolved. 20 ml. of 10 per cent sodium hydroxide are added and 33 g. of sodium chloride. The mixture is again cooled to 30°, with stirring, and the precipitate filtered and washed, as before, with 5 per cent sodium chloride. The washing is repeated twice, filling the funnel each time with the sodium chloride solution; followed by a single washing with distilled water. The purified sodium picrate is again dissolved in a beaker of boiling water, and filtered through a folded filter paper. To the solution are now added 100 ml. of concentrated hydrochloric acid with vigorous stirring. The picric acid crystallizes out; and after cooling to room temperature is filtered on a Buchner funnel, on which it is washed with a few changes of distilled water until free from chloride. The picric acid so prepared is dried at room temperature and preserved in a brown bottle.

Standards. 0.5 ml. (+0.5 ml. water) and 1 ml. of the dilute standard phosphate solution (≡0.002 and 0.004 mg. P) *plus* 1 ml. of 5 per cent trichloroacetic acid.

Blank. 1 ml. of water *plus* 1 ml. trichloroacetic acid.

Test, standards and 'blank' are treated with 3 ml. of water, 0.8 ml. of the acid ammonium molybdate and 0.2 ml. of the reducing agent. The contents of the tubes are gently shaken after each addition, and the colours are read in a colorimeter after 10 minutes [orange (607) light filter; see p. 203].

CALCULATION

Photoelectric Colorimeter.

(1) Low standard :—

$$\text{Blood phosphate}^* \begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.002 \times \dfrac{100}{0.04} \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 5 \end{cases}$$

(2) High standard :—

$$\text{Blood phosphate}^* \begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.004 \times \dfrac{100}{0.04} \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 10 \end{cases}$$

* mg. per 100 ml. of blood.

Duboscq Colorimeter. The calculations are the same as above, except that the ratio of readings is inverted to Reading of standard/Reading of test (see p. 196).

SOLUTIONS

Trichloroacetic Acid Solution. 5 g. of pure trichloroacetic acid dissolved in water, and diluted to 100 ml. and mixed.

Acid Ammonium Molybdate. 5 g. ammonium molybdate are added to a mixture of 75 ml. distilled water and 15 ml. of concentrated sulphuric acid in a 100 ml. volumetric flask. The mixture is shaken until dissolution is complete and cooled to room temperature. The solution is then made up to 100 ml. and mixed.

Reducing Agent. 'Stock' stannous chloride : 10 per cent $SnCl_2$ in 5 N-hydrochloric acid (1 : 1 dilution of concentrated reagent

HCl). Reducing agent : a freshly prepared 1 in 10 dilution of the 'stock' with 10 per cent (v/v) sulphuric acid.

Standard Phosphate. A stock solution is made by dissolving 2.194 g. of pure potassium dihydrogen phosphate (KH_2PO_4) in water and making to 500 ml. This solution contains 1 mg. P per ml. A *dilute standard solution* is made by diluting 2 ml. of the stock solution to 500 ml. with water. 1 ml. of this solution contains 0.004 mg. P. Both solutions should be kept saturated with chloroform to prevent any bacterial growth, which might cause a loss of inorganic phosphate.

GLUCOSE

Titrimetric Method for 'True Sugar'

Harding's (1932, 1933) modification of the Schaffer-Hartmann method. This method gives 'true sugar values' as opposed to total reducing substances when applied to the filtrate of unlaked blood described below (King *et al.*, 1937*a*).

PRINCIPLE

The 'sugar' in whole blood is a mixture of glucose, present mainly in the plasma, and nitrogenous reducing substances (chiefly glutathione) in the corpuscles. It is possible to exclude the corpuscles by mixing the blood with isotonic sodium sulphate solution, in which the corpuscles remain intact. A determination of the reducing power then becomes equivalent to an estimation of glucose alone. For normal (fasting) individuals values of 68–96 mg. per 100 ml. are found.

A protein-free filtrate of blood, and alkaline copper reagent are heated together. Part of the copper is thereby reduced and is equivalent to the amount of reducing substance (sugar) present. The degree of reduction is determined indirectly by reoxidizing the reduced copper with iodine and determining the amount of iodine consumed in the process. The reagent contains potassium iodate, from which iodine is liberated by the addition of potassium iodide and sulphuric acid. The iodine set free both in the test and in a 'blank' determination (2 ml. of copper reagent and 2 ml. of water), is determined by titration with sodium thiosulphate. The

difference between the 2 titrations represents the amount of iodine consumed in reoxidizing the reduced copper.

METHOD

The blood is taken into a dilute copper solution, in which there is no loss of sugar by glucolysis (King, Pillai and Beall, 1941). 0.2 ml. of capillary blood is washed from a pipette into 3.5 ml. of isotonic sodium sulphate-copper sulphate solution. One of the Somogyi (1931) protein precipitating reagents (copper) is thus used for the preservation of the blood. When it is desired to complete the analysis, 0.3 ml. of 10 per cent sodium tungstate is added ; the copper tungstate formed precipitates the protein. The mixture is shaken and then centrifuged.

Test. 2 ml. of the supernatant liquid (≡0.1 ml. of blood) are treated with 2 ml. of the mixed copper reagent, accurately measured, in a wide ($\frac{3}{4}$ inch) test tube.

A 'blank' is prepared in a similar test tube with 2 ml. of distilled water and 2 ml. of reagent.

Both tubes, stoppered lightly with cotton wool, are placed in a boiling water bath for exactly 10 minutes. They are then cooled at once under the tap. To each is added 1 ml. of 2 per cent potassium iodide and 1 ml. of 2 N-sulphuric acid. After standing 1 minute the contents of each tube are titrated with N/200 sodium thiosulphate. 1 per cent soluble starch [made up in saturated salt (NaCl) solution, or in saturated phenol red solution] is used as indicator. The titration figure of the test solution is subtracted from that of the 'blank.' The titration of the 'blank' is usually about 7.8 ml. ; that of a 116 mg./100 ml. 'test' would be 6.8 ml. (see below).

CALCULATION

1 ml. N/200 thiosulphate = 0.116 mg. glucose.

The ml. of thiosulphate given by the difference between the 'blank' and 'test' titrations is equivalent to the amount of glucose present in the 'test.' Hence :—

ml. N/200 thiosulphate × 0.116 = mg. glucose in 2 ml. filtrate (i.e. in 0.1 ml. blood)

And therefore :—

$$\left.\begin{array}{r}\text{ml. N/200 thiosulphate} \\ \times 0.116 \times \dfrac{100}{0.1}\end{array}\right\} = \text{mg. of glucose per 100 ml. blood}$$

i.e.,

$$\left.\begin{array}{r}\text{ml. N/200 thiosulphate} \\ \times 116\end{array}\right\} = \text{mg. of glucose per 100 ml. blood}$$

If the blood-sugar value thus obtained is greater than 400 mg./100 ml., the determination should be repeated, using as test solution a mixture of 0.5 ml. of filtrate and 1.5 ml. of water. The result then obtained is multiplied by 4.

It must be emphasized that each worker should establish the 'glucose factor' for himself. The 116 figure will be approximated, but not necessarily reached, if the composition of the reagent is that specified ; but each new batch of reagents should be calibrated against a solution of pure dry glucose, e.g. of 116 mg./100 ml., diluted 1 in 20, and 2 ml. used in the test.

SOLUTIONS

Isotonic Sodium Sulphate-Copper Sulphate Solution (to prevent glucolysis). A mixture of 320 ml. of 3 per cent sodium sulphate ($Na_2SO_4.10H_2O$) and 30 ml. of 7 per cent copper sulphate ($CuSO_4.5H_2O$).

Sodium Tungstate. 10 g. per 100 ml.

Copper Reagent (Harding's modification of the Schaffer-Hartmann reagent).*

Solution A consists of :—

13 g. Copper sulphate crystals dissolved in water and the volume made to 1 litre.

Solution B is made by dissolving :—

24 g. Rochelle salt (sodium potassium tartrate),
40 g. Anhydrous sodium carbonate,
50 g. Sodium bicarbonate,
36.8 g. Potassium oxalate, and exactly
1.4 g. Potassium iodate.

* This solution is very similar to the Somogyi copper reagent, but, in our hands, has been more sensitive, and the results more closely proportional.

Solution B is best prepared as follows :—

The bicarbonate is weighed out, washed into a litre flask with 700 ml. of water and dissolved at room temperature. The carbonate is now added, and shaken until dissolved. The oxalate is weighed into a beaker, dissolved in 120 ml. of warm water, and added to the main solution. The Roehelle salt is dissolved in 100 ml. of water in a beaker, and added to the mixture ; finally, the iodate is weighed out and washed direetly into the solution, which is well shaken, made to the mark, and again thoroughly mixed. (Solution B may form a deposit on standing. This does not affect its oxidizing properties, and may be ignored.)

The 'copper reagent' used in the above method is a freshly made mixture of exaetly equal volumes of solutions A and B.

NOTE. Only purest analytical ehemieals should be used in making up the above reagent.

2 *per cent Potassium Iodide.*

2 N-*Sulphuric Acid.* 54 ml. concentrated H_2SO_4 per litre.

N/200 *Thiosulphate.* Freshly diluted (25 ml. to 500 ml.) standard 0.1 N-sodium thiosulphate (p. 189). This solution is best kept and used in the automatic burette employed for micro-Kjeldahls.

GLUCOSE

Colorimetric Method for 'True Sugar' in 0.05 ml. of Blood

PRINCIPLE

With this method (Haslewood and Strookman, 1939 ; King and Garner, 1947) glucose is estimated accurately in pure solution and in blood. The results obtaincd are identical with those found with the previously described titration method. The proteins are precipitated by sodium tungstate and copper sulphate (Somogyi, 1931) and the filtrate is treated with a modified Harding and Downs copper reagent, from which the iodate is omitted. The cuprous oxidc formed is estimated by the blue colour produced with either a phosphomolybdic acid solution (Folin and Wu, 1920), or with an arseno-molybdic acid solution (Nelson, 1944), which yield almost identical results.

METHOD

Test. 0.05 ml. of whole blood is pipetted into 1.85 ml. of isotonic sodium sulphate-copper sulphate solution in a

conical centrifuge tube. 0.1 ml. of sodium tungstate is added, and the mixture is well shaken. The precipitated proteins and copper tungstate are spun down in the centrifuge. 1 ml. of the supernatant fluid (≡0.025 ml. of blood) is mixed with 1 ml. of the mixed copper reagent in a $\frac{3}{4}$ in. diameter test tube.

Standard. 1 ml. of the standard glucose solutions in benzoic acid is treated in the same way as the blood filtrate.

Blank. 1 ml. water and 1 ml. copper reagent.

The tubes, stoppered with cotton wool, are placed in a boiling water-bath for exactly 10 minutes. After immediate cooling, 3 ml. of the arseno-molybdic acid reagent, or of the phosphomolybdic, are added, and 5 ml. of water (10 or 15 ml. for very densely coloured 'tests'). The red (608) or orange (607) light filter should be used in measuring the colours.

CALCULATION

Photoelectric Colorimeter.

Let X = concentration of standard in mg. per ml.

$$\text{Blood sugar*} = \frac{\text{Reading of test}}{\text{Reading of standard}} \times X \times \frac{100}{0.025}$$

e.g., with 0.02 mg. per ml. standard Blood sugar*

$$= \frac{\text{Reading of test}}{\text{Reading of standard}} \times 0.02 \times \frac{100}{0.025}$$

$$= \frac{\text{Reading of test}}{\text{Reading of standard}} \times 80$$

Similarly, with 0.05 mg. per ml. standard, Blood sugar*

$$= \frac{\text{Reading of test}}{\text{Reading of standard}} \times 200$$

* mg. per 100 ml. blood.

Duboscq Colorimeter. The calculations are the same as above, except that the ratio of readings is inverted to Reading of standard/Reading of test (see p. 196).

SOLUTIONS

Solution A. 13 g. $CuSO_4.5H_2O$ are dissolved in water and the volume made to 1 litre.

Modified Solution B. 50 g. $NaHCO_3$ are dissolved in a beaker with stirring in the minimum amount of distilled water (about

700 ml.). When all the biearbonate is dissolved, 40 g. anhydrous Na_2CO_3 are added with stirring to the solution. When the earbonate has dissolved, a solution of 36.8 g. potassium oxalate in 120 ml. of warm water is added to the mixture. Finally, a solution of 24 g. sodium potassium tartrate in the minimum amount of water (about 100 ml.) is added. The mixture is poured and washed into a 1 litre volumetrie flask, the volume is made to the mark, and the solution well shaken.

The *eopper reagent* used is prepared freshly eaeh day and is a mixture of exactly equal volumes of solutions A and B.

Phosphomolybdie Aeid Reagent (Folin and Wu, 1920). 35 g. molybdie aeid and 5 g. sodium tungstate are dissolved in 250 ml. of 2N-NaOH and the solution boiled for 30 minutes. Water is added to about 350 ml., followed by 125 ml. of 89 per eent phosphorie aeid (sp. gr. 1.75) and the volume made to 500 ml.

Arseno-molybdic Aeid Reagent (Nelson, 1946). 25 g. ammonium molybdate are dissolved in 450 ml. of water; 21 ml. eone. sulphurie aeid added, mixed, and then 3 g. of sodium arsenate ($Na_2HAsO_4.7H_2O$) dissolved in 25 ml. of water. The mixture is kept in the 37° bath for 2 days, and is preserved in a brown bottle. 1 volume of this reagent is diluted with 2 volumes of water for use. (Only A.R. ehemieals should be used for the reagent.)

Isotonie Sodium Sulphate-Copper Sulphate Solution. A mixture of 320 ml. of 3 per eent sodium sulphate ($Na_2SO_4.10H_2O$) and 30 ml. of 7 per eent $CuSO_4.5H_2O$.

Sodium Tungstate. 10 g. per 100 ml., kept in a waxed bottle.

Stock Glueose Solution. 0.1 g. pure anhydrous glueose is dissolved in saturated benzoie aeid solution (0.3 per eent) and the volume made to 100 ml. This is a permanent standard.

Standard Glucose Solutions. These are prepared by diluting 2, 3, and 5 ml. of the stoek glueose solution to 100 ml. with saturated benzoie aeid solution; giving standards of 0.02, 0.03 and 0.05 mg. per ml. (equivalent to 80, 120 and 200 mg. glueose per 100 ml. of blood).

ACETONE BODIES

See p. 94.

GALACTOSE

Galactose is not a normal eonstituent of the blood. It is present in eonsiderable amount after oral or intravenous administration, and measurement of the rate at which it disappears from the blood is used as a test of liver function (King and Aitken, 1940), and of thyrotoxicosis (Barnes and King, 1942).

PRINCIPLE

The glucose can be completely removed from diluted blood by fermentation with washed baker's yeast. Galactose remains unattacked and can be estimated in the deproteinized filtrate of the blood by the copper reduction method used in the estimation of blood glucose.

METHOD

Fresh baker's yeast (2 g.) is well washed by shaking with distilled water (10 ml.) in a centrifuge tube. The yeast is spun down; stirred up with a fresh 10 ml. of water, well shaken, and again centrifuged. The washing is repeated a third time. The yeast is finally stirred and shaken with 10 ml. of isotonic sodium sulphate and is ready for use.

Test. The blood sample (0.2 ml.) is added to 2.5 ml. of a mixture of 22 ml. of isotonic sodium sulphate and 3 ml. of 10 per cent sodium tungstate in a centrifuge tube. 1 ml. of yeast suspension is added. The contents of the tube are thoroughly mixed with the aid of a glass rod, and the tube is then incubated in a water thermostat at 37°C. for 15 minutes.

The proteins are precipitated by the addition of 0.3 ml. of 7 per cent copper sulphate. The proteins and the yeast are filtered off or centrifuged (for 5 min.) and the galactose determined in 2 ml. of the supernatant fluid (equivalent to 0.1 ml. of blood) by the titration method described for blood glucose.

Blank. A blank determination consisting of 2.7 ml. of isotonic sulphate-tungstate and 1 ml. of yeast suspension is carried through at the same time as the test. The blank determination on the yeast should give the same figure in ml. of thiosulphate as is given by the reagent blank determination with water, which is always carried out in the sugar determination. The yeast should also be tested against pure glucose and against galactose solutions. 0.2 ml. of a 100 mg. per 100 ml. galactose solution is analysed with and without the addition of yeast. The same reducing power should be found in both filtrates. With the glucose solution, after

treatment with yeast, no reduction of the copper reagent should be found, i.e. the titration should equal that of the reagent blank with water. (No preservative should be used in preparing these sugar solutions.)

CALCULATION

The factor 162, multiplied by the difference between the ml. of thiosulphate used in test and blank, gives the mg. of galactose per 100 ml. of blood. (The factor for galactose should be determined independently by each worker with his own set of reagents.)

SULPHONAMIDES

Sulphanilamide appears in the blood in variable amount after administration. Part of it is present in the free state and part in a conjugated form. For therapeutic purposes it is usual to try to adjust the amount administered by mouth so as to maintain a level of 8–15 mg. of the free sulphanilamide per 100 ml. of blood. The total sulphanilamide (i.e. free plus conjugated) is usually 20–50 per cent more than the free.

PRINCIPLE

This method is adapted from the procedure of Bratton and Marshall (1939). The blood is deproteinized with trichoroacetic acid. Sulphanilamide is estimated in the filtrate by means of the diazo reaction. The sulphanilamide is diazotized with sodium nitrite, the excess nitrite is destroyed by ammonium sulphamate, and the diazonium compound is coupled with naphthyl ethylene diamine. The pink colour produced is compared with that developed from a standard sulphanilamide solution treated in the same way. Some sulphonamide may be conjugated, e.g. as glucuronide or 'ethereal sulphate,' and must be released from combination before it will react with the reagents. This is accomplished by boiling the trichloroacetic acid filtrate with hydrochloric acid. Determination of the sulphanilamide in the resultant solution gives the total sulphanilamide, free plus conjugated.

The chemically related drugs, sulphapyridine and sulphathiazole, are determined in the same way.

METHOD

0.2 ml. of blood is added to 3.2 ml. of water or isotonic sodium sulphate ; 0.6 ml. of 25 per cent trichloroacetic acid is added. The mixture is vigorously shaken, and filtered or centrifuged.

Test. 2 ml. of the filtrate (≡0.1 ml. of blood) are transferred to a test tube and 1 drop of sodium nitrite solution added. The tube is shaken and left for 3 min. ; 1 ml. of ammonium sulphamate solution is added and the mixture left for 2 min. with occasional shaking. 2 ml. of naphthyl ethylene diamine solution are now added and the mixture shaken.

Standard. A standard is prepared in the same way from 2 ml. of standard solution (≡0.004 mg. sulphanilamide).

Blank. 1.8 ml. water, 0.2 ml. trichloroacetic acid, 1 drop sodium nitrite, 1 ml. ammonium sulphamate and 2 ml. naphthyl ethylene diamine. A yellow-green light filter (e.g. Ilford 625) is used for the comparison.

Total sulphanilamide is determined by heating 2 ml. of filtrate with 0.5 ml. of N-hydrochloric acid in a 5 ml. volumetric flask in a boiling-water bath for 1 hour. The cooled contents of the flask are then treated with sodium nitrite, etc., as in the procedure for free sulphanilamide, and the volume adjusted to 5 ml. with water.

CALCULATION

Photoelectric Colorimeter.

$$\text{Blood sulphanilamide*} \begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.004 \times \dfrac{100}{0.1} \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 4 \end{cases}$$

* mg. per 100 ml. blood.

Duboscq Colorimeter. The calculation is the same as above, except that the ratio of readings is inverted to Reading of standard/Reading of test (see p. 196).

If the colour of the test is more than twice as strong as that of the standard, the determination should be repeated with 1 ml. of filtrate plus 1 ml. of water.

If sulphapyridine or sulphathiazole is determined by the above procedure, and with a sulphanilamide standard, the value obtained is multiplied by a factor to give the mg. sulphapyridine or sulphathiazole per 100 ml. blood. Factors for these and other sulphonamides are calculated from the ratios of their molecular weights to that of sulphanilamide and are given in Table 4.

TABLE 4

Conversion Factors for the Common Sulphonamides in Terms of Sulphanilamide, Sulphapyridine and Sulphathiazole

Substance to be determined	Standard substance used		
	Sulphanilamide	Sulphapyridine	Sulphathiazole
Sulphanilamide . . .	—	0.69	0.68
Sulphapyridine . . .	1.45	—	0.98
Sulphathiazole . . .	1.48	1.02	—
Sulphamethylthiazole . .	1.56	1.08	1.05
Sulphadiazine . . .	1.45	1.00	0.98
Sulphadimethylpyrimidine .	1.62	1.12	1.09
Sulphaguanidine . . .	1.35	0.93	0.91
Sulphanilylsulphanilamide .	1.90	1.31	1.28
Sulphasuccinylsulphathiazole	2.06	1.43	1.39
Sulphamethazine . .	1.3		

These are derived from considerations of the molecular weights and are mostly in good agreement with the determined figures.

SOLUTIONS

25 *per cent Trichloroacetic Acid.* 25 g. of the acid dissolved in water and made to 100 ml.

Sodium Nitrite Solution. 0.5 g. dissolved in 100 ml. of water. This solution is the same as solution B in the bilirubin method.

Ammonium Sulphamate Solution. 0.5 g. dissolved in water and made to 100 ml.

Naphthyl Ethylene Diamine Solution. 0.05 g. of *N*-1-naphthyl ethylene diamine dihydrochloride in 100 ml. of water; stored in a brown bottle.

Stock Standard Sulphanilamide (0.1 mg. per ml.). 100 mg. of sulphanilamide are dissolved in 1 litre of water.

Standard Sulphanilamide Solution (0.002 mg. per ml.). 2 ml. of the stock standard, together with 15 ml. of the 25 per cent trichloroacetic acid, are diluted to 100 ml. with water.

HÆMOGLOBIN

Ammundsen (1939, 1941) has shown that a normal adult may have from 2 to 12 per cent of his total hæmoglobin circulating in an inactive form. Most methods of estimating hæmoglobin are standardized by reference to the oxygen-carrying power of blood. This takes no account of inactive hæmoglobin which may be capable of regeneration to the active form. For this reason it is preferable to utilize the iron content of blood as a measure of its hæmoglobin content. Blood contains about 50 mg. of iron per 100 ml. and almost all of this is present in the form of hæmoglobin. Only a fraction of a mg. of non-hæmoglobin iron is present in the plasma. Since all forms of hæmoglobin contain iron in the same proportion, its estimation serves to give the total hæmoglobin content of the blood. Moreover, all colorimetric methods agree much more consistently with iron analyses of blood than they do with oxygen capacities (King *et al.*, 1948).

Authorities differ as to the percentage of hæmoglobin in blood which they regard as a normal figure. The three most commonly used are given in Table 5.

TABLE 5

Concentrations of 'Normal' Blood Standards

	Haldane	Haden	Sahli
g. hæmoglobin per 100 ml. . .	14.8*	15.6	17.2
O_2-capacity (ml. per 100 ml.) .	19.8	20.9	23.0
mg. Fe per 100 ml. . . .	49.4	52.2	57.6

* See King *et al.* (1947).

Accurate Determination of Hæmoglobin by Iron Analysis of Blood

Test. Blood (5 ml.) is washed with distilled water from a pipette calibrated to contain into a 50 ml. pyrex beaker, a few drops of concentrated nitric acid are added and the beaker is capped with filter paper. The mixture is cautiously evaporated to dryness and then heated more strongly until fuming ceases. The beaker is placed overnight in a muffle furnace at 400°C., the residue dissolved by warming with 5 ml.

concentrated hydrochloric acid, and the solution diluted to 20 ml. with water. The indicator (1 ml. of 20 per cent potassium thiocyanate) is added and the contents titrated with undiluted commercial titanous chloride ($TiCl_3$) to a colourless end-point.

Standard. 5 ml. of the dilute iron standard (≡0.0025 mg. Fe).

The $TiCl_3$ is contained in a 'micrometer syringe burette' (Burroughs Wellcome & Co.), which consists of a horizontally

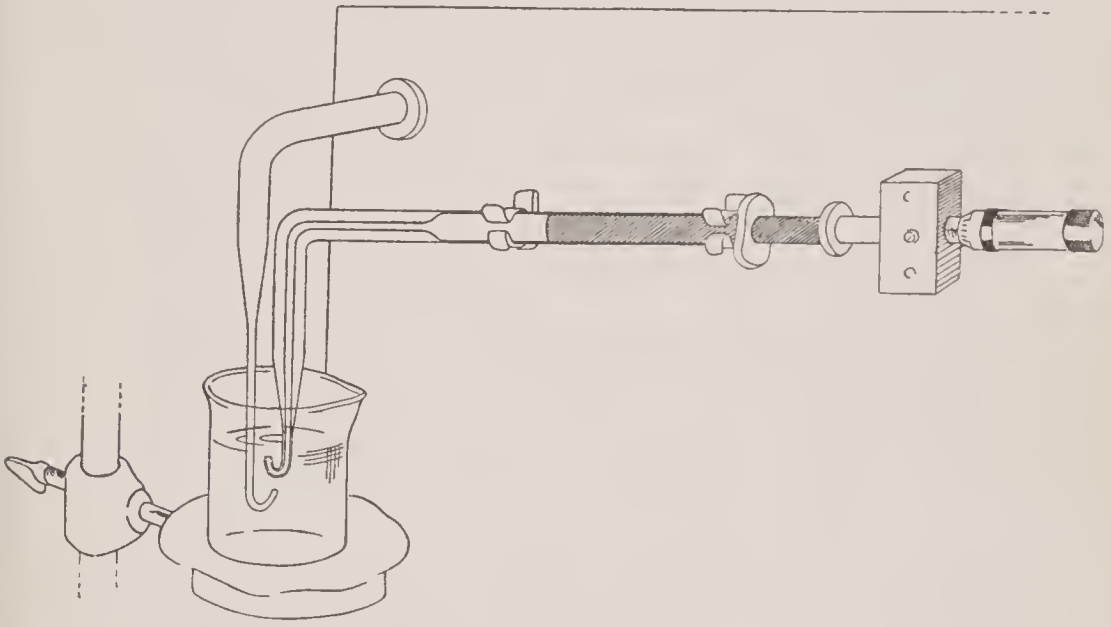


Fig. 1. Micrometer syringe burette.

mounted all-glass 1 ml. syringe, the plunger being driven by a micrometer screw (Trevan, 1925); the smallest division corresponds to a delivery of 0.0002 ml. The fused-on glass capillary outlet has a turned-up tip to minimize diffusion. It dips into the iron solution together with a tube delivering carbon dioxide for continuous stirring. The $TiCl_3$ in the burette, which is sufficient for 6–8 titrations, does not deteriorate over a period of an hour, and titrations can be made slowly to a stable end-point. The glass parts of the apparatus may be replaced by a tuberculin syringe with a fused-on capillary outlet. Titrations of the blood solutions are alternated with titrations of standard iron solution washed from the same pipette to avoid calibration errors.

CALCULATION

$$\text{Blood hæmoglobin (g. Hb per 100 ml.)} \begin{cases} = \dfrac{\text{Titration of test}}{\text{Titration of standard}} \times 0.0025 \times \dfrac{100}{5} \times \dfrac{100}{0.334} \\ = \dfrac{\text{Titration of test}}{\text{Titration of standard}} \times 15 \end{cases}$$

$$\text{Blood hæmoglobin (as per cent of Haldane normal)} \begin{cases} = \dfrac{\text{g. Hb per 100 ml.} \times 100}{14.8} \end{cases}$$

NOTE. There is 0.334 per cent of iron in hæmoglobin, and there are 14.8 g. of hæmoglobin in 100 ml. of normal blood on the Haldane scale.

SOLUTIONS

Concentrated Nitric Acid. Iron-free concentrated HNO_3.

Concentrated Hydrochloric Acid. Iron-free concentrated HCl.

Thiocyanate. 20 per cent (w/v) KCNS.

Titanous Chloride. Commercial concentrated (15 per cent) $TiCl_3$ solution.

Iron Standard. The stock iron standard is an acid solution of ferric ammonium sulphate (43.18 g. dissolved in water with about 100 ml. concentrated HCl and made to 1 litre) containing 5 mg. Fe per ml.; it is standardized by iodimetric titration against $Na_2S_2O_3$ (Treadwell and Hall, 1930). The dilute iron standard is a 1 : 10 dilution of this, standardized by $TiCl_3$ titration against a solution of 99.7 per cent pure iron wire. The two methods of standardization agree within 0.2 per cent.

Colorimetric Methods

PRINCIPLE

Oxyhæmoglobin. A measured quantity of blood is suitably diluted with water, and the intensity of the red colour is measured photometrically with a green light filter.

Carboxyhæmoglobin. The solution of hæmoglobin is treated with carbon monoxide from a cylinder or generator, or with coal gas which contains carbon monoxide. The cherry-red colour of the carboxyhæmoglobin, so produced, is matched colorimetrically against a standard solution, or photometrically with a green light filter.

Cyanhæmatin. The hæmoglobin solution is treated with hydrochloric acid which turns most forms of hæmoglobin into the pale yellow acid hæmatin. The cyan derivative of hæmatin, produced by adding sodium cyanide, is intensely red-coloured. The intensity of the colour is easily matched colorimetrically against a standard solution of cyanhæmatin, or photometrically with a green light filter.

Alkaline Hæmatin. The hæm—the pigmented constituent of the hæmoglobin molecule—is split off from the protein part (globin) by treatment with alkali. The process may be accelerated by heat or by a preliminary treatment with acid. The brown solution of alkaline hæmatin, so produced, is compared with a standard solution of crystalline hæmin in sodium borate, or with an artificial standard.

PROCEDURES

Oxyhæmoglobin. *Test.* 0.02 ml. of blood is diluted to 4 ml. with dilute ammonia solution (0.4 ml. concentrated NH_3 to 1 litre); or 0.05 ml. to 10; or (for more accurate work) 0.5 ml. to 100 ml.

Standard. The intensity of the red colour is determined by reading the solution in the M.R.C. photometer (p. 36), or in a Duboscq or photoelectric colorimeter by reference to a grey screen or grey solution to which a hæmoglobin value has been assigned by the National Physical Laboratory (obtainable from Messrs. Keeler Ltd., 39 Wigmore Street, London, W.1). An Ilford 625 yellow green light filter should be used.

At the 1 in 200 dilution used and with the 625 filter, a normal blood (of 14.8 g. hæmoglobin per 100 ml., i.e. 100 per cent Haldane) has an extinction coefficient of 0.475. A neutral grey screen of 0.475 Density (Ilford or Chance) should, therefore, be used as a 100 per cent standard, and grey screens of other Density accordingly, e.g. a 0.38 Density screen=80 per cent when the hæmoglobin solution is read in a suitable colorimeter at a depth of 1 cm. Thomson's (1946) grey solution may also be used in a sealed test tube as a standard, and, at the dilution of 2 parts Thomson's grey and 1 part water, has an optical density of 0.475, and is equal to 100 per cent when compared with hæmoglobin in a similar test tube. It has been found, however, that colorimeters and 625 light filters differ sufficiently, one from another, for this

relation not to be always strictly observed. It is advisable, for accurate work, to have each combination of grey screen or grey solution, light filter and colorimeter calibrated by the National Physical Laboratory or Messrs. Keeler (who supply the combination of light filter and grey screen to fit any colorimeter).

Carboxyhæmoglobin. By employing photoelcetric colorimeters (EEL, Gallenkamp, Hilger, etc.) and mieroadaptors which fit the B.S.I. Haldane standard and test tube, results are obtained much more easily and quickly than is possible by the dilution technique, which agree well with the most accurate colorimetric procedures and with iron and gas analyses (King and Geiser, 1950).

A Haldane standard and test tube, marked only at the 2 ml. mark (100 per cent), are obtainable from Messrs. Hawksley and Son, New Cavendish Street, London, W.1. (The factor for conversion to true hæmoglobin percentages of the ratio of test to standard readings is established by submission of the standard and test tube to the National Physieal Laboratory, Teddington.)

Test. Blood (0.02 ml.) is washed from a capillary pipette into about 1 ml. of water (containing 0.4 per cent of concentrated ammonia solution) in the test tube, and is saturated with carbon monoxide or with coal gas. Water (0.4 per eent ammonia) is added earefully to the mark and the mixture gently shaken.

Standard. The readings (extinctions) are then taken, of the test and the standard, in the photoeleetric colorimeter fitted with a bright speetrum yellow-green light filter (Ilford 625) and the mieroadaptor for the Haldane tubes.

Calculation. The ratio of the readings (Test/Standard) is multiplied by 100 and by the N.P.L. factor to give the eoneentration of hæmoglobin in percentage of Haldane normal, or by 14.8 × the factor to give the hæmoglobin in grams per 100 ml. of blood.

Cyan Hæmatin. *Macro Method.* 0.5 ml. of blood is treated in a 100 ml. volumetric flask with 50 ml. N/10 HCl and left till the transformation to aeid hæmatin appears to be complete, e.g. 5 to 10 minutes. Thereupon 2 per eent sodium cyanide in N/10 sodium hydroxide solution is added to

the mark. The colour is read against the cyan hæmatin standard with a yellow-green light filter (625).

Standard. A solution of 28.8 mg. of crystalline hæmin (8.57 per cent iron) in 1 litre of 1 per cent sodium cyanide has the same colour as 14.8 g. of hæmoglobin per 100 ml. (Haldane 100 per cent normal) when treated by the above method. Samples of hæmin of other than theoretical iron content should be used proportionately, e.g. 30 mg. of hæmin of 8.2 per cent iron ($28.8 \times 8.57/8.2 = 30$). Crystalline hæmin is obtainable from British Drug Houses, Poole, Dorset.

Micro Method. 0.05 ml. blood is added to 4.95 ml. N/10 HCl, left for 5 to 10 minutes, and then 5 ml. of 2 per cent sodium cyanide in N/10 sodium hydroxide added.

Alkaline Hæmatin. *Method* 1. 0.05 ml. of blood is diluted with 4.95 ml. of N/10 NaOH, heated in a boiling-water bath for 4 to 5 minutes, cooled, and read against the hæmin standard in a suitable colorimeter (photoelectric, Duboscq, etc.), using a green light filter.

Method 2. (Method of Wu; cf. Peters and Van Slyke, 1932). 0.05 ml. of blood is treated with 4 ml. of N/10 HCl, left at room temperature for 40 minutes, and then diluted to 5 ml. with N-NaOH.

Alkaline Hæmatin Standard. Crystalline hæmin (75 mg. of pure hæmin of 8.57 per cent iron), dissolved in 100 ml. of sodium borate buffer, kept 2 days in the ice box, and then diluted to 1 litre with borate buffer pH 9.4,* gives the same intensity of colour as blood of 14.8 g. hæmoglobin per 100 ml. (100 per cent Haldane) when diluted 1 in 100 with N/10 sodium hydroxide, and compared in a photoelectric or visual colorimeter with a green light filter (624). Samples of hæmin of other than theoretical iron content should be used proportionately, e.g. 76.5 mg. of a hæmin which analyses 8.41 per cent of iron ($75 \times 8.57/8.41 = 76.5$). New standards should be prepared semi-yearly and kept in a cool place. Crystalline hæmin is obtainable from British Drug Houses, Poole.

Artificial Standard for Alkaline Hæmatin. Gibson and Harrison's (1945) standard is permanent and very convenient; and is obtainable from British Drug Houses. It is a solution of chromium potassium sulphate [11.61 g. $CrK(SO_4)_2.12H_2O$], anhydrous cobalt sulphate (13.10 g. $CoSO_4$) and potassium bichromate

* Borate buffer pH 9.4: 900 ml. of 0.05 M-borax (19.07 g. $Na_2B_4O_7.10H_2O$ per litre) *plus* 100 ml. 0.1 N-sodium hydroxide.

(0.690g. $K_2Cr_2O_7$) in 500 ml. water, with 1.8 ml. N-H_2SO_4, heated to boiling for 1 min., cooled and made to 1 litre with distilled water.

The volume 4.95 ml. is best measured by means of specially calibrated pipettes. These are easily prepared by selecting good 5 ml. delivery pipettes and allowing 0.05 ml. to run from the tip through a short, fine rubber connexion into a 0.05 ml. capillary pipette. The position of the meniscus is then noted, slightly below the original mark on the 5 ml. pipette, and a new mark is made with a file or diamond.

CALCULATION

Photoelectric Colorimeter.

$$\text{Hæmoglobin}^* \left\{ = \frac{\text{Reading of test}}{\text{Reading of standard}} \times 14.8^{\ddagger} \right.$$

$$\text{Hæmoglobin}^{\dagger} \left\{ = \frac{\text{Reading of test}}{\text{Reading of standard}} \times 100^{\ddagger} \right.$$

* g. per 100 ml.

† As per cent of Haldane normal.

‡ Multiplied by any factor attached to the standard solution, grey screen or grey solution.

Duboscq Colorimeter. Calculation same as above, except that the ratio of readings is inverted to Reading of standard/Reading of test (see p. 196).

M.R.C. Photometer. When hæmoglobin estimations, performed by the oxyhæmoglobin procedure, are read with the yellow-green filter (625) the result is obtained directly as percentage on the scale. The value obtained is the same as would be found by carrying out the Haldane determination using the B.S.S. calibrated colour-tube, diluting pipette, etc. On this scale, 100 per cent=14.8 g. hæmoglobin per 100 ml. of blood (not 13.8 g. Hb./100 ml.; see King *et al.*, *Lancet*, 1947, ii, 789). Conversion from percentage to grams of hæmoglobin can be carried out by multiplying the reading by 14.8/100 (see pp. 32, 99).

Chapter III

PROCEDURES FOR PLASMA

TAKING OF BLOOD FOR PLASMA

With a *dry* syringe blood is taken from an arm vein in the usual way. The arm should be warm ; if a tourniquet is applied to distend the vein, it should be used immediately before the puncture is made and removed as soon as blood begins to flow. 5–10 ml. of blood are taken and allowed to flow gently into a test tube or small jar which has been dried in an oven at about 100°C. after the addition of 1 drop of a 30 per cent solution of potassium oxalate. The blood is thoroughly mixed, by repeated inversion (not violent shaking), with the oxalate. If the plasma is needed for CO_2-combining power or chloride analysis, the blood must be centrifuged immediately, and in any case the plasma should be separated and pipetted off from the cells as soon as possible, without chilling of the blood.

BILIRUBIN

Normal blood contains small amounts of the yellow pigment bilirubin. There is no generally accepted range of normal values. By this method they fall within the range 0.1 to 0.8 mg. per 100 ml. of plasma, with the majority of the values within the limits 0.3 to 0.5 (Vaughan and Haslewood, 1938 ; Wootton, Maclean-Smith and King, 1950). These quantities may be greatly increased in various types of jaundice.

PRINCIPLE

The plasma is treated with diazotized sulphanilic acid, with the addition of ammonium sulphate and alcohol to precipitate the protein. The red colour produced was originally compared colorimetrically against a standard solution of bilirubin, treated with diazotized sulphanilic acid. Bilirubin is, however, difficult to obtain pure, and various artificial 'permanent standards' have been devised. The most satisfactory

is that containing methyl-red (*o*-carboxybenzeneazodimethylaniline—2.9 mg. per litre at pH 4.63) in sodium acetate buffer. The colour of this solution accurately matches the colour obtained when 0.04 mg. of bilirubin is treated with the diazo reagent in a final volume of 10 ml.

METHOD

Test. 1 ml. of plasma or serum is treated in a centrifuge tube (or, better, in the glass-stoppered tube described on p. 40) with 0.5 ml. of diazo reagent. If the diazo reagent is carefully 'layered' above the plasma, and the tube allowed to stand for a few moments, a positive 'direct' van den Bergh reaction (if present) may be seen at the liquid junction. 0.5 ml. of saturated ammonium sulphate and 8 ml. of 85 per cent ethyl alcohol are added. The mixture is stoppered, thoroughly mixed, allowed to lie on its side for 30 minutes, and filtered. Under these conditions the dilution of the plasma closely approximates to 1 in 10.

Standard. The colour of the clear filtrate is compared with the standard mentioned above (≡0.04 mg. of bilirubin in a volume of 10 ml.) (Haslewood and King, 1937; King and Coxon, 1950). The comparison is made with the green light filter 624 (see p. 203).

If the concentration of azo-bilirubin in the test appears to be more than twice that in the standard, a suitable dilution of the original plasma with the phosphate buffer solution should be made, and the procedure repeated. Since this involves a dilution of the plasma (e.g. 1 in 3, 1 in 10), the resultant reading must be multiplied by the dilution factor. Obviously icteric plasma should be diluted in the first instance.

CALCULATION

Photoelectric Colorimeter.

$$\text{Bilirubin}^* \begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.04 \times \dfrac{100}{1} \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 4 \end{cases}$$

* mg. per 100 ml. of plasma.

Duboscq Colorimeter. It is frequently found that brownish or purplish tints produced in the reaction make the colorimetric comparison with the standard solution difficult. These extraneous colours (probably due to traces of substances other than bilirubin which react with the diazo reagent) may be eliminated by the use of a green light filter (see section of photometric measurement, p. 197). The green filter (Ilford 624 green is an appropriate type) is placed over the eye-piece of the colorimeter and the reading made against the artificial standard. The adjustment is then made in a green field whose two halves are of exactly the same quality of colour and differ only in intensity. The reading is taken in the usual way.

The calculation is the same as above, except that the ratio of readings is inverted to Reading of standard/Reading of test (see p. 196).

SOLUTIONS

Stock Standard Methyl-red Solution. 290 mg. of pure methyl-red are dissolved in 100 ml. of glacial acetic acid.

Methyl-red Standard (2.9 mg. per litre at pH 4.63). 1 ml. of the above standard is placed in a litre flask, together with 5 ml. of glacial acetic acid. Water is added, and 14.4 g. of crystallized sodium acetate are washed into the flask. When dissolution is complete, the volume is made to 1 litre with water.

Diazo Reagent. This is made by mixing two solutions, A and B.

Solution A is made by dissolving 1 g. of sulphanilic acid in 250 ml. of N-hydrochloric acid, and making the volume to 1 litre with water.

Solution B contains 0.5 g. of sodium nitrite in 100 ml. of aqueous solution.

The diazo reagent mentioned above is made freshly before use by mixing 0.3 ml. of solution B with 10 ml. of solution A.

Alcohol. 85 per cent.

Buffer. 3.6 g. of disodium phosphate ($Na_2HPO_4.12H_2O$) in 100 ml. water.

Ammonium Sulphate. Saturated solution.

CHOLESTEROL

(Based on the method of Sackett, 1925)

Total Cholesterol

Total cholesterol in the blood of normal persons may be present in amounts varying from 140 to 215 mg. per 100 ml.

The amounts present may be raised in severe diabetes, biliary obstruction, and in some forms of nephritis (particularly the nephrotic type). The concentration of cholesterol is fairly constant in the cells, even in disease, most of the increases being confined to the plasma, on which it is, therefore, better to make the determination than on whole blood.

PRINCIPLE

Blood plasma is treated with an alcohol-ether mixture, which extracts the cholesterol and at the same time precipitates the proteins. The extract is evaporated to dryness, and the cholesterol in it determined by means of the Liebermann-Burchard reaction. This comprises the production of a green colour when chloroform solutions of certain sterols are treated with acetic anhydride and sulphuric acid.

METHOD

Test. 0.2 ml. of plasma is pipetted drop by drop into a centrifuge tube* containing a mixture of 8 ml. of absolute alcohol and 2 ml. of ether. The tube, closed with a dry stopper, is shaken vigorously for about 1 minute, and is then allowed to lie horizontally, with an even distribution of the precipitate along the tube, for 30 minutes. The mixture is then centrifuged, and the supernatant liquid poured, as completely as possible, into a small beaker. This is placed on a water-bath or hot plate (low temperature), and the contents carefully evaporated to dryness. The residue is washed out with about 4 ml. of chloroform, in 3 portions, into a glass-stoppered 10 ml. measuring cylinder, and the volume made to 5 ml. with chloroform.

Standard. 0.5 ml. (≡0.5 mg. of cholesterol) of the cholesterol standard is added to a similar container and diluted to 5 ml. with chloroform.

Blank. 5 ml. of chloroform.

To each solution are added 2 ml. of acetic anhydride, and 0.1 ml. of concentrated sulphuric acid. The cylinders are stoppered, and their contents mixed and allowed to stand in

* Glass-stoppered tubes are preferable for this determination. A suitable size is $4\frac{1}{2} \times \frac{5}{8}$ in., with a B.10 standard ground-glass top and stopper.

the dark for 10 minutes. The solutions are then read in the colorimeter. [Red (608) or orange (607) light filter.]

CALCULATION

Photoelectric Colorimeter.

$$\text{Total cholesterol}^* \begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.5 \times \dfrac{100}{0.2} \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 250 \end{cases}$$

* mg. per 100 ml. of blood.

Duboscq Colorimeter. The calculation is the same as above, except that the ratio of readings is inverted to Reading of standard/Reading of test (see p. 196).

Cholesterol Standard. 100 mg. of pure cholesterol in 100 ml. of chloroform.

PLASMA PROTEINS

The total quantity of protein in blood plasma varies in normal individuals from about 6.3 to 8.2 g. per 100 ml.† Plasma protein is divided into two main fractions: globulin and albumin. Globulin includes fibrinogen. Normally, the approximate amounts of the proteins in plasma are albumin 4.0–5.7 g. per 100 ml.; globulin (excluding fibrinogen) 1.5–3.0 g. per 100 ml.; fibrinogen 0.2–0.4 g. per 100 ml. Where there is decrease of plasma protein—e.g. through proteinuria or malnutrition—the albumin is chiefly affected, and there is often a reduction of the albumin-globulin ratio (normally 1.3–4.0). A reduction of this kind is characteristic of nephrosis. An increase in the globulin, especially fibrinogen, may accompany inflammatory conditions.

Nesslerization Method

PRINCIPLE

Oxalated plasma diluted with isotonic sodium chloride is used for estimation of total protein. Another portion of the

† g. Protein × 2.43 = (approx.) 15–20 milli-equivalents per litre.

diluted plasma is treated with calcium chloride, and the fibrin clot removed. A further (fresh) sample of plasma is treated with sodium sulphite solution, which precipitates the 'globulin,' and the filtrate is used for estimation of 'albumin.' For total protein, and 'albumin,' the protein is precipitated with zinc sulphate and sodium hydroxide, the precipitates and the fibrin clot being then digested with sulphuric acid and selenium dioxide. The protein nitrogen is estimated colorimetrically, as ammonium sulphate, with Nessler's solution. The nitrogen figures multiplied by 6.25 give the approximate protein values, which are expressed as grams per 100 ml. of plasma.

METHOD

(A) Total Protein. *Test.* 0.2 ml. of plasma (from oxalated blood) is diluted to 20 ml. with 19.8 ml. of isotonic (0.9 g. per 100 ml.) sodium chloride. 0.5 ml. of this solution (≡0.005 ml. of plasma) is pipetted into 1 ml. of dilute zinc sulphate and 1 ml. of 0.05 N-sodium hydroxide is added with mixing. The sides of the tube are washed down with 2 ml. of water, and the precipitate is centrifuged down.*

When the supernatant liquid has been carefully decanted, the inverted tube is drained on a filter paper. 0.2 ml. of 50 per cent sulphuric acid containing 1 per cent of selenium dioxide is added. The mixture is gently heated until the water has been driven off, and blackening occurs and white acid fumes appear.† Heating is continued until the mixture is colourless, and for 30 or 40 minutes more. To the cooled

*(A combined centrifuge-digestion tube, 4 × $\frac{7}{16}$ in. with a slight constriction $\frac{3}{4}$in. from the bottom, is useful for this separation and acid digestion; and is obtainable from Messrs. Gallenkamp.)

† An electric heater of the type supplied by Messrs. Gallenkamp, Sun Street, London, E.C. 2, is useful for this purpose. This is best operated: front row of heaters at low and back row at high until the water has all been driven off; and, thereafter, front row at high and back row off until the digestion is complete (Fig. 2; King, 1951).

If no electric heater is available, it is convenient to heat the tubes on a wire and asbestos gauze over a bunsen burner. The tube is slipped into a wire paper clip, the arms of which have been pulled apart to an angle of about 90°, and sufficiently bent for the tube to enter. By placing the clip about an inch from the top of the tube it is then possible to stand it on the gauze at an incline. The bottom of the tube should be at the centre of the gauze. Heat should be applied gently until all the water is driven off, and then more strongly until the digestion is complete.

colourless solution are added 5 ml. of water, and the mixture is well shaken. 3 ml. of Nessler's solution are added and the mixture again well shaken.

Standard. The colour is compared with a standard prepared from 5 ml. of the standard ammonium chloride solution

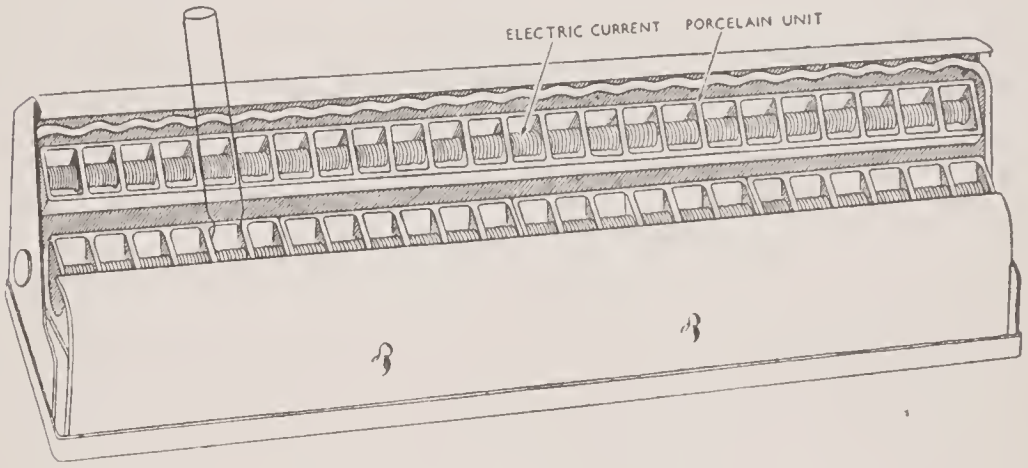


FIG. 2. Electric heater for micro-Kjeldahl and other acid digestions.

(containing 0.01 mg. of nitrogen per ml.) and 3 ml. of Nessler's solution.

Blank. 5 ml. water and 3 ml. Nessler.

A violet (621), blue (622) or blue green (623) light filter should be used in this colorimetric comparison. For photoelectric instruments the filter should be selected which gives a reading in the region of $0.5E$; this will probably be the violet for instruments with glass cells of 0.5 cm. optical depth: blue for 1 cm. and blue green for 1.5 cm. cells (see pp. 203, 206).

CALCULATION

Photoelectric Colorimeter.

$$\text{Total protein (A)*} \begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.05 \times \dfrac{100}{0.005} \times \dfrac{6.25}{1000} \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 6.25 \end{cases}$$

* g. per 100 ml. of plasma (× 2.43 = milli-equivalents per litre).

Duboscq Colorimeter. The calculation is the same as above, except that the ratio of readings is inverted to Reading of standard/Reading of test (see p. 196).

NOTE. If the highest accuracy in the total protein estimation is not necessary, the 0.5 ml. of diluted plasma may be digested with sulphuric acid without the preliminary precipitation of the protein with zinc hydroxide. The results will be approximately 0.2 per cent too high. 0.2 per cent protein corresponds to the average non-protein nitrogen equivalent of 32 mg. per 100 ml. of normal plasma. This simplification should only be used in cases where the N.P.N. is known not to be elevated.

(B) 'Albumin.' 0.2 ml. of plasma is diluted to 10 ml. with 9.8 ml. of a solution of sodium sulphite. The mixture is kept at room temperature for 10 minutes and is then filtered through a fine filter paper (Whatman 32 or 42). 0.5 ml. of the filtrate (≡0.01 ml. of plasma) is placed in 1 ml. of dilute zinc sulphate in a Pyrex centrifuge tube and the protein precipitated with 1 ml. of 0.05 N-sodium hydroxide, spun, drained, digested, and estimated as ammonium sulphate exactly as in the case of total protein.

CALCULATION

Photoelectric Colorimeter.

$$\text{'Albumin' (B)*} \begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.05 \times \dfrac{100}{0.01} \times \dfrac{6.25}{1000} \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 3.125 \end{cases}$$

* g. per 100 ml. of plasma.

Duboscq Colorimeter. The calculation is the same as above, except that the ratio of readings is inverted to Reading of standard/Reading of test (see p. 196).

(C) 'Fibrin.' To 0.1 ml. of plasma, diluted with 4 ml. of isotonic sodium chloride, is added 0.2 ml. of calcium chloride solution. The mixture is kept at 37°C. until clotting occurs, preferably overnight. The fibrin is carefully collected on a thin glass rod and the liquid poured off; the clot is pressed

on the side of the tube to remove liquid, and washed with water, which is likewise poured off. 0.2 ml. of the sulphuric acid mixture is added for digestion, the glass rod with adherent fibrin being left in the tube. This, and also the colorimetric estimation, is carried out exactly as in the case of total protein.

CALCULATION

Photoelectric Colorimeter.

$$\text{'Fibrin' (C)*} \begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.05 \times \dfrac{100}{0.1} \times \dfrac{6.25}{1000} \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.3125 \end{cases}$$

* g. per 100 ml. of plasma.

Duboscq Colorimeter. The calculation is the same as above, except that the ratio of readings is inverted to Reading of standard/Reading of test (see p. 196).

$$\textbf{(D†) 'Globulin'} \begin{cases} = \text{Total protein} - (\text{'albumin'} + \text{'fibrin'}) \\ = \text{A} - (\text{B} + \text{C}) \end{cases}$$

† g. per 100 ml. of plasma.

SOLUTIONS

Dilute Zinc Sulphate. 1 in 10 dilution of 10 per cent $ZnSO_4.7H_2O$ (p. 10).

0.05 N-*Sodium Hydroxide.* 1 in 10 dilution of 0.5 N-NaOH (p. 11).

50 *per cent Sulphuric Acid, containing* 1 *per cent of Selenium Dioxide* (SeO_2), *Nessler's Reagent*, and *Standard Ammonium Chloride* as for urea and non-protein nitrogen (pp. 10, 12).

Calcium Chloride. 2.5 g. per 100 ml. in water.

Sodium Sulphite. 42 g. of sodium sulphite ($Na_2SO_3.7H_2O$) dissolved in warm water and made to 100 ml.

It has been shown by Campbell and Hanna (1937) that treatment of plasma with sodium sulphite, instead of sodium sulphate (Howe, 1921) solution, enables the 'globulin' fraction to be precipitated in 10 minutes (instead of 3 hours) and at room temperature (instead of at 37°C). This method, modified for use with 0.2 ml. of plasma, has been found, with bloods containing normal and abnormal ratios of albumin and globulin, to give results almost identical with those obtained by the sodium sulphate procedure previously described.

PLASMA PROTEINS

Kjeldahl Method

PRINCIPLE

The plasma proteins are precipitated by molybdic acid and the precipitate is digested with sulphuric acid. By this process the protein nitrogen is turned into ammonium sulphate. The digested mixture is transferred to a distillation apparatus and the ammonia is liberated by adding an excess of sodium hydroxide. The ammonia is distilled by steam and is carried over into an excess of standard sulphuric acid solution. By titration of the excess of standard acid, that amount of it which has been neutralized by the ammonia is determined. From this the percentage of proteins is calculated.

The albumin, and by difference the globulin, is determined in a similar manner in the filtrate from a sample of plasma which has been treated with sodium sulphite solution. Treatment of the solution with sodium sulphite brings about precipitation of the globulin fraction and leaves the albumin in solution.

METHOD

Total Protein. 0.2 ml. plasma (from oxalated blood) is mixed with 5 ml. of water in a round-bottom Pyrex centrifuge tube. To this mixture are added 0.2 ml. of 7.5 per cent sodium molybdate and 0.2 ml. of $\frac{2}{3}$ N-sulphuric acid. The tube is shaken and centrifuged for 5 min. The supernatant fluid is completely decanted off and the tube inverted and allowed to drain on a filter paper. 2 ml. of 50 per cent sulphuric acid (containing 1 per cent selenium dioxide) are added, together with a small piece of carborundum. The mixture is heated on an electric coil heater or with a very small gas flame, and is gently boiled until blackening occurs ; thereafter the heating is continued for about 2 hours. If a condensate of selenium (reddish-brown deposit) forms at the side of the tube, it is returned to the body of the digestion mixture by gentle shaking.

The distillation of the ammonia is performed with the micro-Kjeldahl distillation apparatus shown in Fig. 3. The apparatus is cleaned by passing steam through it for about 20 minutes (see below), and the cooled contents of the digestion tube are then transferred quantitatively into the

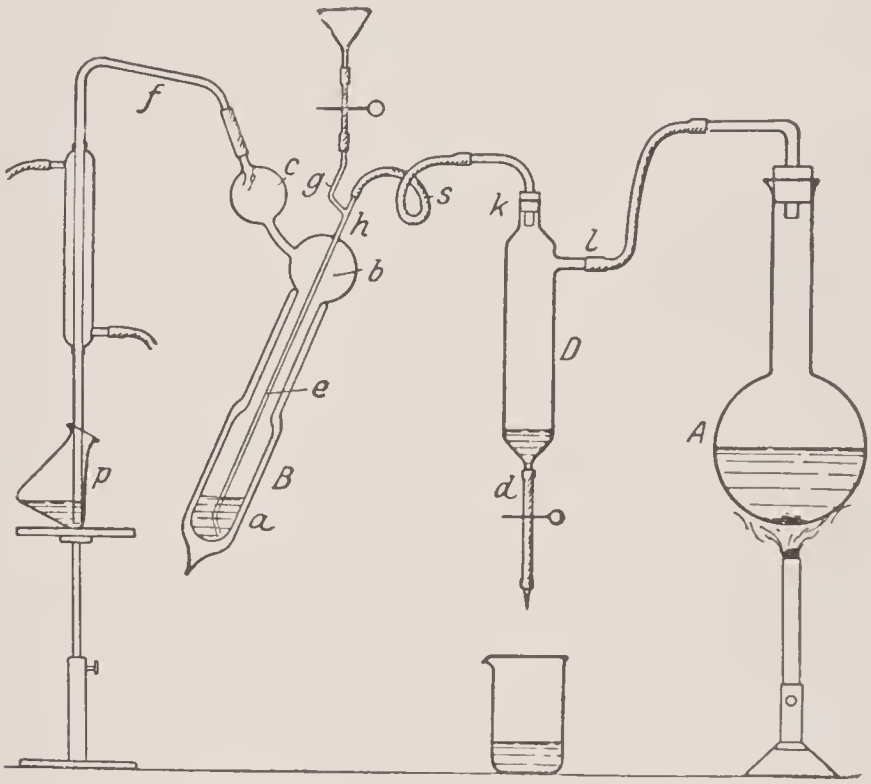


FIG. 3. Parnas and Wagner's improved micro-Kjeldahl apparatus (Pregl).

apparatus, with several washings of water, through the small funnel above *g*.

15 ml. of N/70 sulphuric acid containing the special indicator* are placed in a conical flask, which is put in position with the end of the delivery tube under the surface of the acid (*p*). 10 ml. of concentrated sodium hydroxide (40 per cent) are now added to the distillation apparatus through the small funnel (*g*). The ammonia which is liberated is carried over (through *c* and *f*) into the standard acid by bubbling

* N/70 sulphuric acid in 20 per cent ethyl alcohol, containing 10 ml. per litre of Tashiro's indicator (0.08 per cent methyl red and 0.02 per cent methylene blue in alcohol).

steam through the mixture. A burner is placed under the large round-bottom flask (*A*) which is about half-full of water, and steam is passed (via *l*, *k*, *s* and *e*) through the mixture (*a*) until the amount of liquid in the conical flask is about twice what it was at the beginning of the distillation (10 to 15 minutes). The conical flask is now lowered until the end of the condenser tube (*p*) no longer dips into the liquid ; and the distillation is continued for a minute. The tube is washed down with water, to remove any adhering acid into the conical flask.

The titration of the excess acid in the conical flask is performed from a 10 ml. burette with N/70 sodium hydroxide. The titration figure so obtained is subtracted from the 15 ml. of N/70 sulphuric acid to give the ml. of standard acid which have been neutralized by the ammonia.

Cleaning of Distillation Apparatus. The Bunsen burner is removed from under the boiling flask (*A*) and the partial vacuum thus created causes the liquid in the distillation apparatus to be sucked back into the waste chamber (*D*). When this has occurred the Bunsen burner is replaced and the steam allowed to pass until about 10 ml. of water have accumulated in the distillation chamber (*B*). The Bunsen burner is again removed and the distillate sucks back into the waste chamber which may be drained through *d*. This process is repeated two or three times to ensure thorough cleansing of the distillation chamber. For the precautions which should be observed in performing the micro-Kjeldahl procedure, Pregl's 'Quantitative Organic Microanalysis,' 4th ed., p. 88 (1945), should be consulted.

CALCULATION

1 ml. N/70 H_2SO_4 = 0.2 mg. of nitrogen

$$\text{Total Protein}^* \begin{cases} = \text{ml. N/70 } H_2SO_4\dagger \times 0.2 \times \dfrac{100}{0.2} \times \dfrac{6.25}{1000} \\ = \text{ml. N/70 } H_2SO_4 \times 0.625 \end{cases}$$

* g. per 100 ml. of plasma (× 2.43 = milli-equivalents per litre).

† neutralized by the ammonia.

Albumin. 0.5 ml. of plasma are diluted with 9.5 ml. of 42 per cent sodium sulphite solution. The mixture is well mixed and allowed to stand for 15 minutes, when it is filtered through a Whatman No. 32 or 42 filter paper. 5 ml. of filtrate (≡0.25 ml. of plasma) are transferred to a round bottom Pyrex centrifuge tube. A drop of caprylic (*sec.* octyl.) alcohol and 1 ml. of 50 per cent sulphuric acid are added. The tube is shaken to drive off the SO_2 liberated from the sodium sulphite by the sulphuric acid. 0.5 ml. of 7.5 per cent sodium molybdate is added to precipitate the albumin and the tube is shaken and centrifuged. The supernatant fluid is carefully decanted off and the tube drained. The subsequent analysis is carried out exactly as described for total protein.

CALCULATION

$$\text{Albumin}^* \begin{cases} = \text{ml. N/70 } H_2SO_4\dagger \times 0.2 \times \dfrac{100}{0.25} \times \dfrac{6.25}{1000} \\ = \text{ml. N/70 } H_2SO_4 \times 0.5 \end{cases}$$

* g. per 100 ml. of plasma.

† neutralized by the ammonia.

Fibrin. 1 ml. plasma together with 1 ml. of 2.5 per cent calcium chloride solution and 25 ml. of 0.9 per cent NaCl solution are mixed and kept at 37°C. for 30 minutes or until clotting has occurred. The clot is collected on a thin glass rod, pressed to remove liquid, washed with a little 0.9 per cent NaCl solution and placed in a round-bottom Pyrex centrifuge tube for digestion as in the case of total protein.

CALCULATION

$$\text{Fibrin}^\ddagger \begin{cases} = \text{ml. N/70 } H_2SO_4\S \times 0.2 \times \dfrac{100}{1} \times \dfrac{6.25}{1000} \\ = \text{ml. N/70 } H_2SO_4 \times 0.125. \end{cases}$$

‡ g. per 100 ml. of plasma.

§ neutralized by the ammonia.

VAN SLYKE COPPER SULPHATE METHOD FOR PROTEIN

By Measuring Specific Gravity of Plasma and Serum

PRINCIPLE

Drops of plasma or serum are allowed to fall into graded solutions of copper sulphate of known specific gravity. Each drop on entering the solution becomes encased in a sack of copper-proteins, and remains as a discrete drop without change of gravity for 15 or 20 seconds, during which time its rise or fall reveals its gravity relative to that of the solution. The size of the drops need not be constant, hence no special pipette is required. No temperature correction is needed, because the temperature coefficient of expansion of the copper sulphate solution approximates to that of blood or plasma. The method is capable of measuring gravities to ± 0.00005, which is ten times more than the accuracy required. The copper sulphate solution cleans itself after each test, since the material of the drop settles as a precipitate (Phillips, Van Slyke, Emerson, Hamilton and Archibald, 1945; Hoch and Marrack, 1945; Van Slyke *et al.*, 1950).

METHOD

The plasma may be dropped into the standard bottles (as originally described); but it is preferable to put about 3 ml. of the copper sulphate solutions (starting usually at sp. gr. 1.026) into small test tubes and to discard these after use. By adding a drop of the plasma to each tube, one after the other, that specific gravity in which the drop neither sinks nor rises is quickly determined.

CALCULATION

For determination of protein content an equation is used,

$$\text{Protein}^* = 365\ (\text{Sp. gr.} - 1.007).$$

* in g. protein per 100 ml.

Correction. For heparinized blood (see p. 6) no correction is needed. But the specific gravities for oxalated blood require correcting: for each mg. of ammonium-potassium oxalate mixture added per ml. of blood (see below) 0.0004 should be subtracted from the observed sp. gr. of the plasma. Usually 5 mg. oxalate are used for 5 ml. of blood; and one can neglect the 0.0004 sp. gr. error which leads to an over-estimation of only 0.1 g. protein per 100 ml. of blood.

SOLUTIONS

Stock Copper Sulphate. A stock solution of sp. gr. 1.10 is prepared by dissolving 159.63 g. of $CuSO_4.5H_2O$ per litre at 26.2°. Its specific gravity is checked by weighing 100 ml. in a volumetric flask against distilled water.

Standard Copper Sulphate Solutions are prepared by dilution of the stock. The stock solution and the standard solutions should be prepared within 5° of the same temperature. Once prepared the standard solutions can be used at any temperature within ±15 or 20° of the temperature at which they were made. 100 ml. portions are prepared and stored in 4 oz. bottles.

For the sp. gr. of 1.020, 19 ml. of stock (of sp. gr. 1.10) are measured from a burette into a 100 ml. volumetric flask, and the flask filled with water and the contents transferred to a bottle.

For the sp. gr. of 1.021, 20 ml. stock are similarly diluted; and so on for all sp. gravities, the number of ml. of stock solution less by 1 than the sp. gr. figure in the second and third decimal places being diluted to 100 ml.

For blood plasma and serum the range is usually 1.020 to 1.030. The range can be extended to 1.008 for ascitic fluids and transudates.

Oxalate. For plasma an oxalate mixture of 3 g. ammonium oxalate and 2 g. potassium oxalate in 250 ml. water (≡2 per cent oxalate) is preferable to a simple oxalate. 0.25 ml. of this solution (≡5 mg. oxalate) is dried at about 100°C. in tubes or bottles for 5 ml. samples of blood.

SODIUM

The sodium of the blood is concentrated in the plasma, very little being found in the red cells. In normal persons plasma sodium values lie between 316 and 340 mg. per 100 ml. (137–148 milli-equivalents per litre). In acute cases of Addison's disease the amount is lowered, when values

approaching 250 mg. may be encountered ; in chronic cases it may be only slightly depressed.

PRINCIPLE

After precipitation of the plasma proteins by trichloroacetic acid the sodium in the filtrate is precipitated as sodium zinc uranyl acetate. After washing, this precipitate is treated with ammonium carbonate and hydrogen peroxide and the resulting yellow colour is compared with that produced by a standard sodium chloride solution which has been treated in a similar fashion. This method is adapted from the procedures of Noyons (1939) and Stone and Goldzieher (1949).

METHOD

To 0.5 ml. of serum or plasma are added 1.5 ml. of 7 per cent trichloroacetic acid. The mixture is shaken well and filtered (5.5 cm. paper) after 5 minutes.

Test. 0.2 ml. of the filtrate (≡0.05 ml. plasma) is transferred to a centrifuge tube containing 1 ml. of absolute alcohol and 0.4 ml. of zinc uranyl reagent.

Standard. 0.2 ml. of standard NaCl (≡0.15 mg. Na) is treated simultaneously in the same way as the deproteinized plasma. The contents are mixed and kept in the ice-box overnight ;* they are then centrifuged for 15 minutes. The supernatant solutions are decanted, the tubes allowed to drain on a filter paper for 10 minutes, and the lips dried ; 5 ml. of absolute alcohol saturated with sodium zinc uranyl acetate are added ; the contents are mixed, by rotating the tube, centrifuged for 15 minutes and drained as before. The precipitates are then dissolved in 0.5 ml. of water. 3 ml. of ammonium carbonate and 0.5 ml. of hydrogen peroxide are added and the contents of the tubes diluted to 8 ml.

Blank. 3 ml. ammonium carbonate, 0.5 ml. hydrogen peroxide and 4.5 ml. water.

Colours must be read within 15 minutes. Violet (621) or blue (622) light filter (see p. 203).

* It is not possible to obtain complete precipitation of the sodium zinc uranyl acetate in a short time. A two-hour precipitation may be used, however, although the results will be less accurate.

CALCULATION

Photoelectric Colorimeter.

$$\text{Plasma sodium}^* \begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.15 \times \dfrac{100}{0.05} \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 300 \end{cases}$$

* mg. per 100 ml. of plasma (÷2.3=milli-equivalents per litre).

Duboscq Colorimeter. The calculation is the same as above, except that the ratio of readings is inverted to Reading of standard/Reading of test (see p. 196).

SOLUTIONS

Standard Sodium Chloride (containing 0.75 mg. Na per ml.). 191 mg. of analytical dry sodium chloride dissolved in 100 ml. in water in a volumetric flask.

Trichloroacetic Acid. 7 g. per 100 ml. in water.

Ammonium Carbonate. Saturated solution.

Hydrogen Peroxide. 30 per cent, i.e. '100 vol.'

Zinc Uranyl Acetate Reagent. 20 g. of uranyl acetate, $UO_2(CH_3COO)_2.2H_2O$, 60 g. of zinc acetate. $Zn(CH_3COO)_2.2H_2O$, and 60 ml. of glacial acetic acid are added to 320 ml. of distilled water and warmed gently until dissolved. After standing 24 hours the solution is filtered into a dark bottle and stored in the ice-box. The solution must be filtered immediately before use.

Saturated Alcoholic Sodium Zinc Uranyl Acetate. 40 ml. of zinc uranyl acetate reagent are mixed with 50 ml. of 50 per cent alcohol saturated with sodium chloride; 100 ml. of absolute alcohol are added, and, after standing in the ice-box overnight, the supernatant solution is decanted. The precipitate is washed several times with alcohol, drained, dried and then shaken with 500 ml. of absolute alcohol. This is stored in the ice-box and filtered immediately before use.

CHLORIDE

The blood plasma of normal persons contains from 590 to 620 mg. of chlorides per 100 ml. (expressed as NaCl†). A

† The results may often be advantageously expressed in terms of Cl instead of NaCl. To accomplish this the figure obtained for mg. NaCl should be multiplied by 0.607 (Cl/NaCl=35.5/58.5); or as milli-equivalents, by dividing the mg. NaCl per litre by the molecular weight of sodium chloride (58.5) which gives 101 to 106 milli-equivalents.

decreased plasma chloride may occur in febrile conditions, particularly pneumonia, diabetes, Addison's disease, and in cases of gastro-intestinal disturbances associated with vomiting or with diarrhœa.

Iodimetric Method

PRINCIPLE

The method is based on the reaction :—

$$NaCl + AgIO_3 \longrightarrow AgCl + NaIO_3$$

Silver iodate in ammoniacal solution is added to a measured amount of plasma. The proteins and the excess of silver iodate, together with the silver chloride formed, are precipitated by the addition of a mixture of tungstic acid and phosphoric acid, leaving in solution an amount of soluble iodate equivalent to the amount of chloride originally present. After the addition of potassium iodide, the amount of iodine set free from the soluble iodate is determined by titration with thiosulphate (Haslewood and King, 1936 ; Sendroy, 1937 : Van Slyke and Hiller, 1947 ; King and Bain, 1950).

METHOD

0.2 ml. of plasma is treated with ammoniacal silver iodate reagent (0.5 ml.) and, after mixing, with tungstic-phosphoric acid (3.3 ml.). The mixture is shaken and filtered through a small fine paper (Whatman 42, 7 cm.). 1 ml. of filtrate ($\equiv$0.05 ml. of plasma), with the addition of 1 ml. of 2 per cent potassium iodide, is titrated with 0.005 N-sodium thiosulphate, with starch as indicator.

CALCULATION

Chloride (as mg. NaCl per 100 ml. of plasma) = 97.5 $\times$ titre.

SOLUTIONS

Preparation of Silver Iodate Reagent. Silver iodate may be purchased, or may be prepared by mixing equimolecular solutions of silver nitrate (mol. wt. 170) and potassium iodate (mol. wt. 214). The precipitate is filtered, washed with distilled water, dried in vacuo, and preserved in the dark.

1.8 g. of solid silver iodate are dissolved in 100 ml. N-ammonia (p. 188). Both silver iodate and its ammoniacal solution appear to decompose slightly when kept, with liberation of soluble iodate. Immediately before a series of determinations, therefore, 5 ml. of the stock (1.8 per cent) ammoniacal silver iodate are acidified with N-sulphuric acid (5 ml.) and centrifuged. The supernatant fluid is discarded and the iodate redissolved in 5 ml. of 0.3 N-ammonia.

Tungstic-Phosphoric Acid Reagent. 4.2 g. of sodium tungstate ($Na_2WO_4.2H_2O$) are dissolved in 1 litre of 0.15 M-phosphoric acid. The latter is prepared by diluting 10 ml. of concentrated phosphoric acid (sp. gr. 1.72) to 1 litre.

0.3 N-*Ammonia.* 17 ml. of concentrated ammonia solution (sp. gr. 0.882) are diluted to 1 litre, and standardized by reference to the N-sulphuric acid (pp. 186 and 188).

Mercuric Nitrate Method of Schales and Schales

PRINCIPLE

The method involves the titration of chloride with mercuric nitrate solution. Mercuric ions combine with chloride to give mercuric chloride :—

$$Hg^{++} + 2Cl^- \longrightarrow HgCl_2$$

Mercuric chloride does not form a precipitate ; but it is so slightly dissociated that the end point of the titration can be recognized by the appearance in the solution of mercuric ions on the addition of the first slight excess of mercuric nitrate. These form an intensely violet-blue complex with diphenylcarbazone which is used as indicator (Schales and Schales, 1941).

METHOD

0.2 ml. plasma is pipetted into a small porcelain evaporating basin. 1.8 ml. of distilled water are added, followed by 0.6 ml. of diphenylcarbazone solution. From a 2 ml. burette mercuric nitrate solution is run into the diluted plasma until a pale violet colour is detected. The titre is compared with that obtained for 0.2 ml. of standard NaCl solution.

CALCULATION

$$\text{Plasma chloride (as mg. NaCl per 100 ml.)} = \frac{\text{ml. } Hg(NO_3)_2 \text{ for test}}{\text{ml. } Hg(NO_3)_2 \text{ for standard*}} \times 584.5$$

$$\text{(as milli-equivalents per litre)} = \frac{\text{ml. } Hg(NO_3)_2 \text{ for test}}{\text{ml. } Hg(NO_3)_2 \text{ for standard*}} \times 100$$

* This titre does not vary, and need be made only occasionally as a check.

Notes. *End Point.* With the addition of the first few drops of mercuric nitrate solution to the diluted plasma an initial violet colour may develop. If this be the case, the titration is continued, when the colour will fade and disappear ; only to reappear again when excess of $Hg(NO_3)_2$ has been added. The end point is then taken to be the point at which the violet colour reappears.

Jaundiced Plasma. The above procedure is not recommended for jaundiced plasma, with which the end point is not sharp. For jaundiced plasma a Folin-Wu protein-free filtrate should be prepared (0.5 ml. plasma, 3.5 ml. water, 0.5 ml. 10 per cent sodium tungstate and 0.5 ml. 2/3 N-sulphuric acid ; shaken and filtered), the mercuric nitrate solution being titrated against 2.0 ml. of filtrate (equivalent to 0.2 ml. plasma).

SOLUTIONS

Standard Sodium Chloride Solution (containing 100 milli-equivalents NaCl per litre or 584.5 mg. NaCl per 100 ml.). 584.5 mg. of analytical dry NaCl are dissolved in 100 ml. of water in a volumetric flask.

Mercuric Nitrate Solution. 2.9 to 3.0 g. mercuric nitrate are added to 300 ml. water. 20 ml. 2 N-nitric acid (p. 190) are added and the solution made up to 1 litre with distilled water. This solution keeps indefinitely, and need be standardized initially only against the sodium chloride solution.

Diphenylcarbazone Solution. (0.1 per cent solution in 95 per cent alcohol). 50 mg. diphenylcarbazone are dissolved in 50 ml. 95 per cent alcohol. This solution should be stored in a dark bottle in a refrigerator. It should be prepared fresh every month.

CARBON DIOXIDE COMBINING POWER

Between 56 and 74 ml. of carbon dioxide are normally held (partly in solution and partly in chemical combination) by 100 ml. of blood plasma (25 to 33 milli-equivalents per litre). About 90 per cent is present in the combined form as sodium bicarbonate, and a determination of the amount of carbon dioxide which can be held by a sample of blood plasma gives a measure of the alkali present. The alkali of plasma is thus usually referred to in terms of its 'CO_2-combining power.' In clinical conditions in which an 'acidosis' is present (e.g. diabetes, starvation, and severe nephritis) the production in the body of various acid substances results in part of the alkali of the plasma being combined with these other acid bodies. Less carbon dioxide is consequently held in combination by the plasma, and the 'CO_2-combining power' is therefore reduced. In other clinical conditions involving the accumulation of excess alkali in the blood (e.g. alkali administration, intestinal obstruction) more carbon dioxide is held in combination in the plasma than is normally the case. This condition is usually known as one of 'alkalosis.'

Van Slyke Method

PRINCIPLE

The carbon dioxide is liberated from its combination with alkali in the plasma by the addition of acid and the volume of gas evolved is measured in a special apparatus designed by Van Slyke. The volumetric method and apparatus introduced by Van Slyke and Cullen (1917) is described. This has been found more suitable for student use than the manometric apparatus of Van Slyke and Neill (1924). The latter should be used for work requiring the highest accuracy (cf. Peters and Van Slyke, 1932), but the simpler volumetric apparatus is adequate for all routine purposes.

METHOD

5 ml. of freshly drawn oxalated blood are centrifuged until the supernatant plasma is free of cells. The plasma is then

separated, and transferred to a test tube or small waxed bottle, under a layer of liquid paraffin if the determination is not to be done immediately.

About 2 ml. of plasma are transferred to a separatory funnel (about 250 ml.). By means of a short rubber tube, the stem of the funnel is connected to a bottle containing glass beads and a little water (see Fig. 4).

The stop-cock of the separatory funnel is opened, the stopper removed and a long breath slowly blown into the mouthpiece of the bottle and through the separatory funnel. Immediately the expiration is complete, the stop-cock is closed and the stopper replaced in the separatory funnel. The

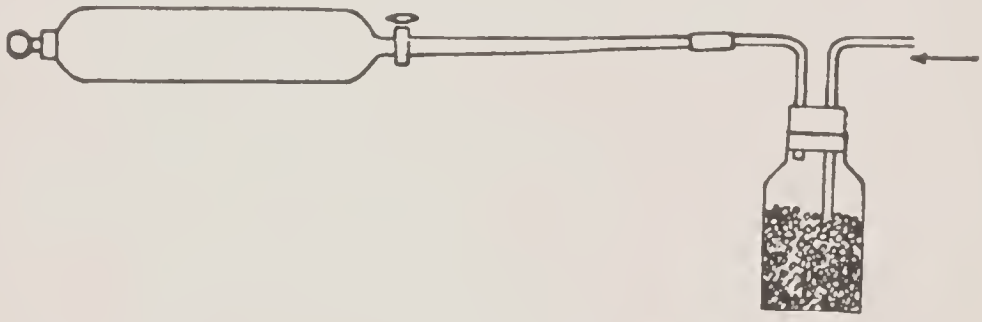


FIG. 4. Apparatus for saturating plasma with CO_2 (Van Slyke). The bottle contains glass beads.
(From Beaumont and Dodds: *Recent Advances in Medicine.*)

plasma is now in contact with a carbon dioxide-containing atmosphere which is very close in composition to alveolar air. After disconnecting the funnel at the rubber tube, the plasma is spread over the walls of the funnel by gentle rotation for 1 min. in order that it may become saturated with the carbon dioxide of the alveolar air. The plasma is now allowed to drain into the narrow end of the funnel.

The Van Slyke apparatus (see Fig. 5) is prepared for use in the following manner.

Stop-cock (F) is opened and the mercury reservoir is raised. The mercury rises in the chamber (A) of the apparatus, and is allowed to flow into the side arm from the stop-cock (E). Stop-cock (E) is now turned to connect with the inlet chamber (B) and the level of mercury raised in order to fill the capillary opening at the bottom of (B). (E) is now turned off and the

apparatus should be air free [e.g. with no bubbles at the top of the tubes (C) and (D) immediately below the stop-cock (F)]. This can be tested for, with the stop-coek (F) open first to eonneet the chamber with (D) and then with (C), by lowering the mereury reservoir until the mercury falls to the 50 ml. mark and then gently raising till the mercury comes with a clicking sound into thc top of the burette below (E). If the mercury does not cliek soundly into the top of the burette, air has leaked into the apparatus and the stop-cocks should be re-greased in order that they shall be airtight.

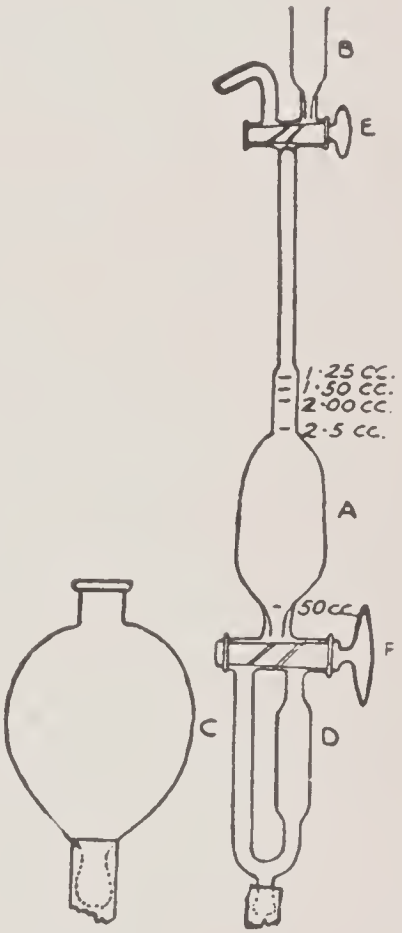


FIG. 5. Volumetric van Slyke apparatus. (After Beaumont and Dodds.)

Distilled water (1 ml.) is added to the inlet chamber (B). 1 ml. of plasma is removed from the separatory funnel and is run into thc chamber (B) below the surfaee of the distilled water. A drop of eaprylie aleohol is added as an anti-frothing agent and the eontents of (B) are allowed to run into the burette by earefully opening the stop-cock (E) and very gradually lowering the mereury reservoir from its previous position at the height of (E). A small amount of water should be left filling the capillary at the bottom of (B). Stopcock (E) is now turned off, great eare being taken that no air is allowed to pass through it into the burette. 0.5 ml. of 10 per cent laetie acid solution is now plaeed in (B) and is carefully drawn into the burette in the manner already deseribed, eare being again taken to leavc a small amount of liquid in the capillary at the bottom of (B) and admitting no air into the burette. The mercury reservoir is lowered until the mercury is at the 50 ml. mark and the stop-cock (F) is turned off. The contents of the burette and 50 ml. chamber are now under a partial

vacuum and the carbon dioxide which has been liberated from its combination as sodium bicarbonate by the lactic acid is seen to boil off from the liquid. In order to bring all the carbon dioxide out of the acidified plasma the apparatus is gently shaken by inversion about 20 times, after carefully removing it from the clamp. After replacing the apparatus, the stop-cock (F) is opened and the plasma mixture very carefully sucked into the chamber (D), leaving a very small amount of fluid at the bottom of the 50 ml. chamber. (F) is now turned round so as to be open to (C), and the mercury is allowed to run into the 50 ml. chamber and up into the graduated burette tube. The surface of the mercury in the reservoir is held level with the surface of the mercury in the burette tube, so that the carbon dioxide in the burette is now at a pressure equal to that of the atmosphere. The volume of the gas is read from the burette and is corrected to standard pressure and temperature. For this calculation the temperature and barometric pressure at the time of the experiment are, of course, necessary.

The apparatus should be thoroughly rinsed with distilled water between determinations ; and occasionally with sodium hydroxide solution, followed by dilute nitric acid, and then several changes of distilled water. Should the glass chambers become dirty with precipitated protein and grease, adherent to their interior, it is advisable to empty the mercury, disconnect the rubber tubing, and leave the glass parts immersed overnight in chromic-sulphuric acid cleaning solution, after which they should be thoroughly washed with tap and distilled water. The mercury can be most easily cleaned by squeezing it several times through a clean cloth towel into a large porcelain evaporating basin.

CALCULATION

The figure obtained, the ml. of carbon dioxide released from 1 ml. of plasma, is reduced by reference to Table 6 to the number of ml. of carbon dioxide which would be liberated from 100 ml. of plasma at standard temperature and pressure. This is the 'CO_2-combining power' of the plasma. The milli-equivalents are obtained by dividing by 2.24.

TABLE 6

Calculation of the CO_2-combining Power of Plasma

(v=measured ml. of CO_2) (p=barometric pressure)

$v\frac{p}{760}$	ml. CO_2 (reduced to 0° and 760 mm.) bound as bicarbonate in 100 ml. of plasma				$v\frac{p}{760}$	ml. CO_2 (reduced to 0° and 760 mm.) bound as bicarbonate in 100 ml. of plasma			
	15°	20°	25°	30°		15°	20°	25°	30°
0.20	9.1	9.9	10.7	11.8	0.60	47.7	48.1	48.5	48.6
1	10.1	10.9	11.7	12.6	1	48.7	49.0	49.4	49.5
2	11.0	11.8	12.6	13.5	2	49.7	50.0	50.4	50.4
3	12.0	12.8	13.6	14.3	3	50.7	51.0	51.3	51.4
4	13.0	13.7	14.5	15.2	4	51.6	51.9	52.2	52.3
5	13.9	14.7	15.5	16.1	5	52.6	52.8	53.2	53.2
6	14.9	15.7	16.4	17.0	6	53.6	53.8	54.1	54.1
7	15.9	16.6	17.4	18.0	7	54.5	54.8	55.1	55.1
8	16.8	17.6	18.3	18.9	8	55.5	55.7	56.0	56.0
9	17.8	18.5	19.2	19.8	9	56.5	56.7	57.0	56.9
0.30	18.8	19.5	20.2	20.8	0.70	57.4	57.6	57.9	57.9
1	19.7	20.4	21.1	21.7	1	58.4	58.6	58.9	58.8
2	20.7	21.4	22.1	22.6	2	59.4	59.5	59.8	59.7
3	21.7	22.3	23.0	23.5	3	60.3	60.5	60.7	60.6
4	22.6	23.3	24.0	24.5	4	61.3	61.4	61.7	61.6
5	23.6	24.2	24.9	25.4	5	62.3	62.4	62.6	62.5
6	24.6	25.2	25.8	26.3	6	63.2	63.3	63.6	63.4
7	25.5	26.2	26.8	27.3	7	64.2	64.3	64.5	64.3
8	26.5	27.1	27.7	28.2	8	65.2	65.3	65.5	65.3
9	27.5	28.1	28.7	29.1	9	66.1	66.2	66.4	66.2
0.40	28.4	29.0	29.6	30.0	0.80	67.1	67.2	67.3	67.1
1	29.4	30.0	30.5	31.0	1	68.1	68.1	68.3	68.0
2	30.3	30.9	31.5	31.9	2	69.0	69.1	69.2	69.0
3	31.3	31.9	32.4	32.8	3	70.0	70.0	70.2	69.9
4	32.3	32.8	33.4	33.8	4	71.0	71.0	71.1	70.8
5	33.2	33.8	34.3	34.7	5	71.9	72.0	72.1	71.8
6	34.2	34.7	35.3	35.6	6	72.9	72.9	73.0	72.7
7	35.2	35.7	36.2	36.5	7	73.9	73.9	74.0	73.6
8	36.1	36.6	37.2	37.4	8	74.8	74.8	74.9	74.5
9	37.1	37.6	38.1	38.4	9	75.8	75.8	75.8	75.4
0.50	38.1	38.5	39.0	39.3	0.90	76.8	76.7	76.8	76.4
1	39.1	39.5	40.0	40.3	1	77.8	77.7	77.7	77.3
2	40.0	40.4	40.9	41.2	2	78.7	78.6	78.7	78.2
3	41.0	41.4	41.9	42.1	3	79.7	79.6	79.6	79.2
4	42.0	42.4	42.8	43.0	4	80.7	80.5	80.6	80.1
5	42.9	43.3	43.8	43.9	5	81.6	81.5	81.5	81.0
6	43.9	44.3	44.7	44.9	6	82.6	82.5	82.4	82.0
7	44.9	45.3	45.7	45.8	7	83.6	83.4	83.4	82.9
8	45.8	46.2	46.6	46.7	8	84.5	84.4	84.3	83.8
9	46.8	47.1	47.5	47.6	9	85.5	85.3	85.2	84.8
0.60	47.7	48.1	48.5	48.6	1.00	86.5	86.2	86.2	85.7

SOLUTIONS

Caprylic Alcohol. Sec. octyl alcohol.

10 *per cent Lactic Acid.* 1 volume of pure concentrated lactic acid diluted to 10 volumes with water.

Capillary Method for CO_2 Combining Power of Plasma

PRINCIPLE

This rapid method, which requires only a small amount of plasma, has been found useful in estimations of the CO_2 combining power as a field method. The plasma, equilibrated with alveolar air, is mixed with an equal quantity of a standard sulphuric acid, and by trial the concentration of acid required to reduce the pH of the mixture to 5.5 is found. This procedure was developed from that described by Wright and Colebrook (1921).

APPARATUS AND SOLUTIONS

Capillator Set for Chlorophenol Red (British Drug Houses).

Standard Sulphuric Acids of various concentrations. These are prepared by dilution of standard N/10 acid, and are preserved in waxed bottles. For most purposes the following series of strengths is sufficient :—

N/20, N/25, N/30, . . . , N/50.

Chlorophenol Red Solution, 0.04 per cent, in aqueous solution.

Light Blue Optical Filter, e.g. Ilford 303.

METHOD

A small Pasteur-type pipette is prepared by fitting a rubber bulb to a capillary tube. It is graduated by marking the tube at about 1 and 2 cm. from the open end, i.e. to contain 1 or 2 volumes. 2 volumes of chlorophenol red solution are withdrawn and discharged into a watch-glass, or on to a waxed slide. Using the same pipette, 1 volume of one of the standard acids, followed by 1 volume of serum or plasma, is withdrawn, added to the indicator and well mixed.

The pH of the mixture is ascertained by drawing it up into the pipette and comparing it with the standard tubes on the card. The comparison is facilitated by viewing the tubes by transmitted light through the blue filter.

After washing the pipette in distilled water the procedure is repeated with another acid until the concentration of acid is found which will give a final pH of 5.5

CALCULATION

CO_2 combining power in ml./100 ml. = 1165 × normality of acid required to reduce pH to 5.5, e.g. if acid was N/35, then CO_2 combining power = 1165 × 1/35 = 33. The figure 1165 is an empirical factor found by comparing titrations with gasometric results. A series of parallel determinations has shown that the standard deviation of the titration results from the gasometric results is ±4 ml./100 ml. (Wootton and King, 1949).

PHOSPHORUS: DISTRIBUTION IN BLOOD

The phosphorus of the blood is present partly as inorganic phosphate and partly in combination with several organic substances. It is distributed as follows :—

	mg. P per 100 ml.	
	Whole blood	Plasma
Inorganic phosphate . . .	2 to 3	2 to 3*
Ester ,, . . .	20 to 30	1 to 2
Lipid ,, . . .	11 to 14	7 to 10
Nucleotide ,, . . .	2 to 3	—
Total ,, . . .	35 to 50	10 to 15

* 1.2 to 1.8 milli-equivalents.

The inorganic phosphate is about equally distributed in cells and plasma. The ester phosphate consists of 'organic salts' of phosphoric acid, which are present, for the most part, in the cells. Lipid phosphate is that contained in the

phosphatides—lecithin, kephalin and sphingomyelin. There is about twice as much lipid phosphate in the cells as in the plasma.

Inorganic Phosphate. The inorganic phosphate of the blood plasma and cells is easily extracted by trichloroacetic acid, which also serves to precipitate the proteins and to destroy the enzymic action which adds to the inorganic phosphate of shed blood by hydrolysis of the phosphoric esters when the blood is allowed to stand. For this reason, estimation of inorganic phosphate should be conducted within an hour or two of taking the blood. In any case, the plasma should be separated promptly from the cells, which contain nearly all of the phosphoric esters. If it is not possible to conduct the analysis on freshly-separated plasma for several hours, it is to be expected that the inorganic phosphate so obtained will be about a mg. higher than it would be if the analysis were made promptly, since this 1 mg. is about the amount of ester phosphate usually present in 100 ml. of plasma.

Plasma from normal persons contains 2 to 3 mg. of inorganic phosphate ; and figures of 3 to 5 mg. are found if the analyses are not done immediately the blood is taken. High figures for inorganic phosphate are found in the acidosis of nephritis and in gas gangrene, when results up to 10 mg. (and even higher) are obtained. In children the inorganic phosphate is higher than in adults ; 4 to 6 mg. being common. Low phosphate figures (1 to 3 mg.) are often encountered in children suffering from rickets.

Ester Phosphate. The phosphoric esters, like inorganic phosphate, are soluble in acid and are present in a filtrate of blood which has been deproteinized with trichloroacetic acid. The inorganic and ester phosphates are known as 'the acid soluble phosphate.' The small amount of ester phosphate present in the plasma (about 1 mg. P per 100 ml.) is thought to be a phosphoric ester of a sugar or related substance. The cells contain 40–60 mg. ester P per 100 ml. Much of it is present as phosphoglyceric acid ; the composition of the remainder is not fully known. Some of these esters are readily hydrolysed with liberation of free phosphate

by the action of the enzymes present in the blood. Consequently, a decrease in the organic phosphate occurs when blood is allowed to stand ; this change is accelerated if the cells be hæmolysed. It is, therefore, essential to conduct the analysis for ester (as well as for free) phosphate on freshly drawn blood.

The ester phosphate is definitely low in rickets. It returns to normal on the addition of vitamin D to the diet. Children suffering from osteomyelitis may have a high ester phosphate of the plasma, with that of the cells unaffected or even decreased. An increase of ester phosphate has been observed in conditions of trauma due to resorption from muscle.

PRINCIPLE

Inorganic phosphate couples with molybdic acid to form a yellow phospho-molybdate ; this can be reduced to give a blue colour which is directly proportional to the amount of inorganic phosphate present. The ester phosphate is not capable of reacting with molybdic acid until after destruction of the organic matter by digestion with hot concentrated perchloric acid, when all the phosphate is converted to the inorganic, reactable form. Its measurement gives the total phosphate, inorganic plus ester, which was originally present in the trichloroacetic acid extract of the blood.

The ester phosphate is obtained from the difference between the inorganic phosphate and the total 'acid-soluble' phosphate.

METHOD

1 ml. of freshly drawn oxalated plasma or whole blood is treated with 9 ml. of 5 per cent trichloroacetic acid. The mixture is well shaken and is filtered after 5 minutes. Analyses for inorganic and total 'acid-soluble' phosphate are carried out on the filtrate as below.

Inorganic Phosphate. *Test.* 5 ml. of the clear filtrate (≡0.5 ml. of plasma or blood) are transferred to a test tube marked at 6 ml.

Standard. In another similar tube are placed 5 ml. of the standard phosphate solution (≡0.02 mg. P).

To each tube is added 0.4 ml. of perchloric acid, 0.4 ml. of 5 per cent molybdate and 0.2 ml. of the reducing agent (and water to the 6 ml. mark). The contents of the tubes are gently shaken between each addition, and finally mixed by inverting and shaking. The colours are read after 10 minutes (red light filter, 608).

CALCULATION

Photoelectric Colorimeter.

$$\text{Inorganic phosphate}^* \begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.02 \times \dfrac{100}{0.5} \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 4 \end{cases}$$

* mg. P per 100 ml. ($\div 1.72$ = milli-equivalents per litre).

Duboscq Colorimeter. The calculation is the same as above, except that the ratio of readings is inverted to Reading of standard/Reading of test (see p. 196).

Total 'Acid-soluble' Phosphate. *Test.* 2 ml. of the trichloroacetic acid filtrate from plasma ($\equiv 0.2$ ml. plasma) or 0.5 ml. of filtrate from whole blood or cells ($\equiv 0.05$ ml. blood) are measured into a test tube of good acid-resistant glass, marked at 6 ml. 0.5 ml. of 60 per cent perchloric acid is added and a small piece of carborundum to prevent bumping. The contents of the tube are heated carefully on an electric heater (see p. 43) or with a microburner (using a very small flame). (Approximately 0.1 ml. of perchloric acid is lost in the heating.) As the contents of the tube become concentrated they turn brown and then, as the temperature rises and the acid begins to fume, they become colourless, the organic matter being completely oxidized in a few minutes. In some cases, where the amount of organic material is large and the oxidization slow, it may be necessary to add a drop of nitric acid or of 30 per cent (100 vol.) hydrogen peroxide ; in this case it will be necessary to continue the heating for 3 or 4 minutes after the mixture has become colourless, in order to drive off the excess of these reagents. The cooled contents

are diluted with 5 ml. of water. 0.4 ml. of 5 per cent molybdate and 0.2 ml. of reducing agent are added to the test.

Standard. At the same time a standard is prepared from 5 ml. of the standard solution (0.02 mg. P), 0.4 ml. of perchloric acid, 0.4 ml. of molybdate and 0.2 ml. of reducing agent.

Test and standards are adjusted (if necessary) to the mark, mixed, and read after 10 minutes, using a red 608 light filter.

CALCULATION

$$\text{Total 'acid-soluble' phosphate*} = \frac{\text{Reading of test}}{\text{Reading of standard}} \times 0.02 \times \frac{100}{x},$$

* mg. P per 100 ml.

where x is the number of ml. of plasma or whole blood represented in the trichloroacetic acid filtrate used in the test.

Duboscq Colorimeter. The calculation is the same as above, except that the ratio of readings is inverted to Reading of standard/Reading of test (see p. 196).

Ester Phosphate=total 'acid-soluble' phosphate *minus* inorganic phosphate.

Lipid Phosphate. Lipid phosphate is often determined in connection with investigations of fat metabolism. Increases have been noted in diabetes and nephritis, where the lipid phosphate is related to the degree of lipemia, and in pregnancy and certain hepatic conditions.

METHOD

A. By Alcohol-Ether Extraction. 0.5 ml. of plasma or whole blood is added drop-wise, with shaking, to a mixture of 5 ml. of absolute alcohol and 1 ml. of ether in a 10 ml. volumetric flask. The mixture is carefully heated in a hot water bath until it is boiling. It is then cooled, made to volume with alcohol and is thoroughly shaken. The mixture is filtered, and 5 ml. of filtrate are carefully evaporated to dryness (in two 2.5 ml. portions to minimize the chance of loss through frothing) in a test tube marked at 6 ml. The phosphate is estimated by digestion with perchloric acid, as in the method for total acid-soluble phosphate.

B. By Trichloroacetic Acid Precipitation. The phospholipids are quantitatively precipitated with trichloroacetic acid, together with the plasma proteins (which themselves contain negligible quantities of phosphorus). An estimation of the total phosphate contained in the trichloroacetic acid precipitate of plasma, therefore, gives the same result for lipid phosphate as an estimate of the phosphate in an alcohol-ether extract (cf. Zilversmit and Davis, 1950).

0.2 ml. of plasma is diluted with 1 ml. of water in a centrifuge tube, and trichloroacetic acid (5 ml. of 5 per cent) is then added slowly with swirling of the contents of the tube. The precipitate is centrifuged down, the supernatant poured off and the tube drained. 0.5 ml. of perchloric acid is added and the tube heated until the digestion is complete. Water, molybdate and reducing agent are added, and the colorimetric determinations completed as for total acid-soluble phosphate.

Total Phosphate in Blood. The whole of the phosphate of the blood can be determined by the colorimetric method after destruction of the organic matter (proteins, fats, etc.) by boiling perchloric acid. For this purpose 0.05 ml. of blood, accurately measured, is digested and the determination carried out as in the total acid-soluble phosphate method.

SOLUTIONS

Trichloroacetic Acid Solution. 5 g. of best grade trichloroacetic acid are dissolved in water and made to 100 ml.

Perchloric Acid. Analytical Reagent quality. 60 per cent.

Ammonium Molybdate Solution. 5 g. ammonium molybdate are dissolved in water and made to 100 ml.

Reducing Agent. 0.2 per cent 1 : 2 : 4-aminonaphtholsulphonic acid in 12 per cent sodium meta-bisulphite and 2.4 per cent sodium sulphite (w/v).

The reducing agent may be prepared fresh for use from a tablet containing the correct amounts of 1 : 2 : 4-aminonaphtholsulphonic acid, sodium sulphite and sodium meta-bisulphite. A tablet is ground with 10 ml. of water, and the filtered solution is ready for use. (Obtainable from Messrs. Gallenkamp.)

Ascorbic acid may also be used as the reducing agent, and should be prepared fresh for use by dissolving one 50 mg. tablet in 25 ml. of water.

Stock Standard Phosphate. A stock solution is made by dissolving 2.194 g. of pure potassium dihydrogen phosphate (KH_2PO_4) in

500 ml. in water. This solution contains 1.0 mg. P per ml. (p. 20).

Standard Phosphate Solution is made by diluting 2 ml. of the stock solution to 500 ml. with water. This solution contains 0.02 mg. P per 5 ml. (0.004 mg. per ml.). Both solutions should be kept saturated with chloroform to prevent any bacterial growth, which might otherwise cause a loss of inorganic phosphate.

ALKALINE PHOSPHATASE IN SERUM OR PLASMA

Phosphatase is the name given to an enzyme shown to be present in bone and ossifying cartilage by Robison (1923). He has demonstrated that this enzyme is intimately related with the proeess of bone formation. The blood plasma of normal individuals contains small amounts of phosphatase eorresponding, per 100 ml., to about 5 to 10 'units'—the arbitrary term in which the amount of the enzyme is expressed. In conditions of generalized bone disease the phosphatase appears to leak out of the bone into the blood, and appears there in large amounts. Its quantitative determination in the serum or plasma is of diagnostic value in cases of rickets, Paget's disease, osteitis fibrosa cystica (Kay, 1931) and in many cases of malignant bone disease (Franseen, Simmons and McLean, 1939 ; for review see King and Delory, 1948).

A marked aecumulation of the enzyme in the serum has also been shown to take place in obstructive jaundice (Roberts, 1933 ; Armstrong, King and Harris, 1934 ; Bodansky and Jaffe, 1934). In conditions of toxic and infective jaundice the rise is not so marked, and in hæmolytic jaundice the enzyme is present in normal amount. The range of values encountered elinically in the different types of jaundice is fully discussed by Herbert (1935) ; Cantarow and Nelson (1937) ; Sherlock (1946). Normal persons have 10 or less units per 100 ml. Cases of hæmolytic jaundice show values which are not above the normal. In infective and toxic jaundice, and in obstructive jaundiee where the obstruction is only partial or intermittent, the values are raised above normal and are usually between 10 and 30 units. In obstructive jaundice where the obstruction is eomplete or of long standing, the phosphatase is

greatly increased, values above 30 and sometimes as high as 200 being encountered. In generalized bone disease the values are very similar to those found in obstructive jaundice.

PRINCIPLE

The estimation of phosphatase depends upon measuring the amount of hydrolysis which takes place when the enzyme is allowed to act on a suitable substrate—an ester of phosphoric acid (such as phenyl phosphate or glycerophosphate)—under standard conditions. The amount of phosphate or phenol so liberated may be taken as the measure of the amount of enzyme present. The phenol is more easily determined than the phosphate, and three times as much phenol (by wt.) as phosphorus is set free. The hydrolysis is carried out at the optimum pH of 10 for 15 minutes. The results thus obtained agree very closely with those of the method of King and Armstrong (1934) of which this is a modification, and with the method of Jenner and Kay (1932). The results are expressed in arbitrary 'units' of phosphatase activity.

The King-Armstrong 'unit' of phosphatase is defined as the amount of the enzyme which will set free 1 mg. of phenol in the given time under the conditions of the test ;* and hence 'units' per 100 ml. = mg. of phenol set free from the phenyl phosphate under the standard conditions. (In terms of the liberated phosphate, the number of units is equal to three times the mg. P set free.)

METHOD

Phosphatase Estimation by Determination of Liberated Phenol

Test. In a conical centrifuge tube are placed 2 ml. of buffer and 2 ml. of substrate. The tube is allowed to remain in a water-bath at 37°C. for 3 minutes. Without removal of the tube from the bath, exactly 0.2 ml. of plasma (which must be cell-free) is added and mixed. The stoppered tube is allowed to remain in the bath exactly 15 minutes. At the end of this time 1.8 ml. of dilute Folin-Ciocalteu phenol reagent are added and the mixture centrifuged or filtered.

* The Bodansky unit is one-third to one-half the King-Armstrong unit.

Control. In another tube are placed 2 ml. of buffer and 2 ml. of substrate. 1.8 ml. of dilute Folin-Ciocalteu reagent are added, followed by 0.2 ml. of plasma and the mixture centrifuged or filtered.

4 ml. of filtrate from the test and control solutions are pipetted into test tubes. 2 ml. of 15 per cent sodium carbonate are added and the tubes replaced in the water-bath for 10 minutes to bring up the colour.

Standard. The solutions are compared in the colorimeter with a standard made up at the same time by taking 4 ml. of standard-phenol-solution-and-reagent and 2 ml. of 15 per cent sodium carbonate. (If the coloured solutions are to be read in a photoelectric colorimeter they should be diluted to 10 ml.) Red light filter (608, see p. 203).

CALCULATION

Photoelectric Colorimeter. The phosphatase activity of a plasma is expressed as units per 100 ml. and is numerically equal to the mg. of phenol which would be set free from the phenyl phosphate under the standard conditions by 100 ml. of plasma. Thus :—

$$\text{Alkaline Phosphatase (Units per 100 ml. plasma)} \begin{cases} = \dfrac{\text{Reading of (test—control)}}{\text{Reading of standard}} \times 0.04 \times \dfrac{6}{4} \times \dfrac{100}{0.2} \\ = \dfrac{\text{Reading of (test—control)}}{\text{Reading of standard}} \times 30 \end{cases}$$

Duboscq Colorimeter. The test solution is placed on the left-hand side of the colorimeter and set at 30 mm. The standard is placed on the right-hand side and the colours matched. The use of an orange or red filter will be found to increase the ease of colorimetric comparison, particularly with weak solutions.

Units of phosphatase per 100 ml. =
mg. phenol liberated in test } *minus* { mg. phenol in control.

The number of mg. phenol in 100 ml. of plasma in the test and in the control is found by the equation :—

$$\frac{\text{Reading of standard}}{30} \times 0.04 \times \frac{6}{4} \times \frac{100}{0.2}$$

All figures in the above equation cancel out, making it equal to 'Reading of standard.'

More simply then :—

Units of phosphatase per 100 ml. =

Reading of standard against the test *minus* Reading of standard against the control.

SOLUTIONS

Buffer. M/10 Sodium carbonate-bicarbonate ($6Na_2CO_3$: $4NaHCO_3$). 6.36 g. anhydrous sodium carbonate and 3.36 g. sodium bicarbonate are dissolved in distilled water and made to 1 litre.

Substrate. M/100 Disodium phenyl phosphate. 2.18 g. dissolved in 1 litre of water. The solution should be brought quickly to the boil to destroy any organisms, cooled immediately and preserved with a little chloroform.

Folin and Ciocalteu's Phenol Reagent. This is made by dissolving 100 g. of sodium tungstate, $Na_2WO_4.2H_2O$, and 25 g. of sodium molybdate, $Na_2MoO_4.2H_2O$, in 700 ml. of water, contained in a 1500 ml. flask, adding 50 ml. of phosphoric acid (s.g. 1.75) and 100 ml. of concentrated hydrochloric acid. The flask is connected to a refluxing condenser by means of a ground-glass joint or by using a rubber stopper wrapped in tin foil,* and the mixture boiled for 10 hours ; then 150 g. of lithium sulphate, 50 ml. of water and a few drops of bromine are added. The boiling is continued without a condenser for 15 minutes, the golden-yellow solution allowed to cool and diluted to 1 litre.

This reagent (1 volume) is diluted with water (2 volumes) for use.

Sodium Carbonate 15 per cent (w/v). 15 g. of anhydrous sodium carbonate are dissolved in water and made to 100 ml.

Stock Standard Phenol (1 mg. per ml.). 1 g. pure crystalline phenol is dissolved in, and made to 1 litre with 0.1 N-HCl.

Standard-Phenol-and-Reagent (0.01 mg. phenol per ml.). 5 ml. of the stock standard phenol (1 mg. per ml.) are accurately measured into a 500 ml. volumetric flask, 100 ml. of dilute (1 in 3) Folin-Ciocalteu reagent are added and water to the mark. This solution will keep at least six months, if preserved in the ice-chest.

* If tin foil is employed care must be taken to ensure that the solution does not come into contact with the metal.

Phosphatase Estimation by Determination of Liberated Phosphate

The phenylphosphate method of phosphatase estimation (King and Armstrong, 1934) utilizes the determination of enzymically hydrolysed phenol as a measure of the phosphatase activity of blood plasma and tissue extracts. For some purposes it is preferable to determine the liberated phosphate instead of the phenol, e.g. in the study of rickets in children where a knowledge of both the phosphatase and the inorganic phosphate of the plasma is useful. Since, in the hydrolysis of phenylphosphate, equimolecular amounts of phenol and phosphate are liberated, therefore, approximately 3 mg. of phenol (mol. wt. 94) are hydrolysed for each 1 mg. of inorganic phosphorus (at. wt. 31). The King-Armstrong unit of phosphatase is defined as the amount of enzyme which liberates 1 mg. of phenol in 30 min. at pH 9 and 37°C., or in 15 min. at pH 10 and 37° (King, 1947). Using the same conditions of hydrolysis it is consequently necessary to multiply the number of mg. of liberated P by three, in order to obtain the same unitage of phosphatase. The following procedure gives close agreement with phosphatase determinations made by the estimation of phenol according to the phenylphosphate method.

Test. 3 ml. of buffer *plus* 3 ml. of substrate are warmed at 37°C. for 3 min. 0.3 ml. plasma is added, mixed and kept at 37° for 15 min. The tube is removed from the bath and 1.2 ml. trichloroacetic acid (20 per cent) added to stop hydrolysis and precipitate the proteins.

Control. 6 ml. water and 0.3 ml. plasma *plus* 1.2 ml. trichloroacetic acid.

Blank. 3 ml. buffer, 3 ml. phenylphosphate, 0.3 ml. water and 1.2 ml. trichloroacetic acid.

The mixtures are centrifuged or filtered, and 5 ml. filtrate (≡0.2 ml. plasma) are treated with 0.8 ml. of 5 per cent ammonium molybdate in 15 per cent H_2SO_4 (p. 19) and 0.2 ml. aminonaphtholsulphonic acid-reducing agent (p. 68).

Standard. 5 ml. phosphate standard, 0.02 mg. P (p. 69) *plus* 0.8 ml. ammonium molybdate and 0.2 ml. aminonaphtholsulphonic acid.

CALCULATION

Photoelectric Colorimeter (red light filter 608, p. 203).

Phosphatase (units per 100 ml.)

$$= \frac{\text{Reading of test} - (\text{control} + \text{blank})}{\text{Reading of standard}} \times 0.02 \times \frac{100}{0.2} \times 3$$

$$= \frac{\text{Reading of T} - (\text{C} + \text{B})}{\text{Reading of standard}} \times 30$$

Inorganic P (mg. per 100 ml.)

$$= \frac{\text{Reading of control}}{\text{Reading of standard}} \times 0.02 \times \frac{100}{0.2}$$

NOTE. In an alternative micro procedure stannous chloride may be used as the reducing agent for the phosphate estimations. *Test :* 1 ml. buffer, 1 ml. substrate, 0.1 ml. plasma, 15 min. at 37°C., 2.9 ml. trichloroacetic acid (7 per cent). *Control :* 2 ml. water, 0.1 ml. plasma, 2.9 ml. trichloroacetic acid. *Blank :* 1 ml. buffer, 1 ml. substrate, 0.1 ml. water and 2.9 ml. trichloroacetic acid.

A *standard* (1 ml., containing 0.004 mg. P, +1 ml. trichloroacetic acid), and 2 ml. of each of the filtrates, are treated with 3 ml. water, 0.8 ml. of the 5 per cent molybdate in 15 per cent H_2SO_4 and 0.2 ml. of dilute stannous chloride (p. 19).

ACID PHOSPHATASE

The prostate contains a very active phosphatase which, unlike the phosphatase of bone, intestine, kidney, etc., has its pH optimum at an acid reaction. To distinguish it from the better known 'alkaline phosphatase,' this enzyme is called 'acid phosphatase.' There is very little present normally in the blood, but in prostatic conditions, particularly in carcinoma of the prostate with secondaries in the bone, very large amounts may appear in the blood. These are derived in part from the prostate and in part from the secondary growths in the bone. There are normally 1–3 arbitrary acid phosphatase units in 100 ml. of serum or plasma. In cases of carcinoma of the prostate with secondaries, values much

greater than this and up to 30 units and more have been observed (cf. Gutman and Gutman, 1938 ; King and Delory, 1948). A raised alkaline phosphatase, accompanying a high acid phosphatase, confirms involvement of the bones (or liver) ; while normal alkaline, with high acid, phosphatase probably indicates secondaries in the lymph nodes. Acid phosphatase determinations are useful in following the effect of stilbœstrol therapy (cf. Huggins *et al.*, 1941, 1949 ; Watkinson *et al.*, 1944).

PRINCIPLE

The pH optimum of acid phosphatase is between 4.5 and 5 ; whereas that of alkaline phosphatase is between pH 8.4 and 10, depending on the substrate used. Because of this great difference in pH optimum, it is possible to estimate one phosphatase in the presence of the other merely by allowing it to act on the substrate at the characteristic optimum pH. Thus the alkaline phosphatase in blood plasma is inactive at the reaction where acid phosphatase works best ; and vice versa.

The same substrate, i.e. phenyl phosphate, is used for the acid phosphatase as for the alkaline, but citric acid-sodium citrate buffer of pH 4.9 is used instead of the alkaline sodium carbonate buffer.

METHOD

Test and Control. 2 ml. of M/100 disodium phenyl phosphate and 2 ml. of the buffer solution of pH 4.9 are pipetted into each of two test tubes. The tubes are allowed to remain in a 37°C. water-bath for 3 minutes to allow the contents to attain the temperature of the bath. After this time, 0.2 ml. of the plasma is added to one of the tubes and the enzymic hydrolysis allowed to proceed for exactly 1 hour. 1.8 ml. of Folin and Ciocalteu's phenol reagent are then added to each tube and 0.2 ml. of plasma to the control tube. The two tubes are shaken and centrifuged, and 4 ml. of the supernatant pipetted into two test tubes. After the addition of 2 ml. of 15 per cent sodium carbonate, the tubes are replaced in the water-bath for 10 minutes to allow the colours to develop.

Standard. The solutions are read in a photoelectric or Duboscq colorimeter against a standard solution of phenol (i.e. 4 ml. of standard-phenol-and-reagent and 2 ml. of sodium carbonate) which has been similarly treated. The results are expressed in terms of units which are equal to mg. of phenol liberated in 1 hour. The calculation is the same as for alkaline phosphatase.

The colours are rather pale for normal bloods, and for prostate cases where the acid phosphatase is not much elevated. It may be preferable in these instances to use a somewhat longer incubation period, e.g. 3 hours. If this is done the mg. phenol liberated are of course divided by 3 to reduce the figure to 1 hour.

SOLUTIONS

See alkaline phosphatase for all solutions except the buffer.

Citric acid-sodium citrate buffer pH 4.9 is prepared as follows :—

Dissolve 21.0 g. of crystalline citric acid in water, add 188 ml. of N-NaOH, and make to 500 ml. The pH should be checked and adjusted to pH 4.9 if necessary, by dropwise addition of N-NaOH or N-HCl. This solution should be preserved with a few drops of chloroform and kept in the ice-chest.

N.B. Acetate buffer may be used, but citrate is here adopted to make the method conform to Gutmans' 1940 procedure.

FORMALDEHYDE-STABLE ACID PHOSPHATASE

The tissues and fluids of the body contain several phosphatases active at an acid pH (Roche, 1931 ; Kutscher and Wolbergs, 1935 ; Roche, Thoai and Baudoin, 1942 ; Behrendt, 1943). Herbert (1944, 1946) differentiated prostatic phosphatase from that of normal serum by the ready destructibility of the former with alcohol. King, Wood and Delory (1945) found the acid phosphatases of prostate and red cells to be similar in many respects, including easy destruction by ethanol. Red blood cells contain roughly 100 times as much acid phosphatase as is present in serum, and hæmolysis is, therefore, a potent source of error in the estimation of serum acid phosphatase.

PRINCIPLE

Formaldehyde treatment gives a sharp differentiation between the prostatic and red cell acid phosphatases. The latter is completely destroyed by the inclusion of 0.5 per cent neutral formaldehyde in the buffer-phenylphosphate-serum mixture used for the determination; while the prostatic phosphatase is quite unaffected. Other tissue acid phosphatases, including that of normal serum, are variably inhibited by formaldehyde, but none is so sensitive as the red cell enzyme (Abul-Fadl and King, 1949).

A high acid phosphatase after formaldehyde treatment (above 5 units) strongly suggests a prostatic origin (values >3 are 'suspicious'), and in most cases it gives useful information for distinguishing between raised values due to the presence of prostatic phosphatase, and those of different origin. The formaldehyde technique permits the use of hæmolysed sera or plasmas which are otherwise unfit for acid phosphatase determination.

METHOD

Formaldehyde Technique. The procedure is exactly as described above under 'acid phosphatase' except that, in this case, 0.05 ml. formaldehyde reagent is added to the mixture of 2 ml. buffer and 2 ml. substrate solutions.

All the following details are then the same.

Formaldehyde Reagent. Neutral formalin solution (40 per cent) (see p. 127).

PLASMA AMYLASE

The determination of plasma amylase is of importance in the diagnosis of acute pancreatitis in which high values may be obtained. It has the advantage over the estimation of urinary diastase in that it is unnecessary to wait for the collection of a 24-hour specimen of urine. A high plasma amylase is also found in salivary gland inflammation, and low values in liver disease and pancreatic inefficiency. Normal values are 90–163 units per 100 ml.

PRINCIPLE

0.5 ml. of plasma is incubated at 37°C. with 1.5 mg. of starch and the time noted when the mixture no longer gives a blue colour with iodine solution.

The amylase activity is expressed in terms of 'units' of amylase per 100 ml. The 'unit' is defined as the amount of amylase which will destroy 1.5 mg. of starch in 8 minutes.*

METHOD

2 ml. of starch solution are introduced into a test tube which is placed in a water-bath at 37°C. for 2 minutes to allow the contents to attain the temperature of the bath. 0.5 ml. of plasma is added to the tube and a stop-watch started. At intervals of 2–5 minutes 0.2 ml. portions of the reaction mixture are withdrawn and added to previously prepared tubes containing 0.2 ml. samples of iodine solution and the colour observed. The time at which a blue or purple colour is no longer obtained is noted and from this time the amylase activity is calculated. The exact time intervals must depend on experience, since by examination of the colour at each addition it is possible to judge when the next sample should be tested.

CALCULATION

$$\text{Amylase Activity}\dagger = \frac{8}{t} \times \frac{100}{0.5} = \frac{1600}{t}$$

† Units of amylase per 100 ml. of plasma where t is the time taken for the destruction of the starch.

SOLUTIONS

Starch. 75 mg. of starch (dried in a desiccator) are weighed out and made into a paste with a few ml. of cold water. This is poured into a solution of 250 mg. NaCl in 80 ml. of boiling water. The mixture is then cooled and made to 100 ml. 2 ml. of this solution contain 1.5 mg. of starch.

Iodine. Approximately N/200, made by fresh dilution of N/10.

* The unit is defined in this way so that the results become almost identical with those of Somogyi (1941) whose unit is defined in terms of the amount of reducing sugar liberated from starch by plasma under precisely stated conditions.

PLASMA ASCORBIC ACID

The amount of ascorbic acid present in the blood plasma of apparently healthy individuals ranges from about 0.4 mg. to 2 mg. per 100 ml. The amount varies greatly with the nutritional habits of the individual. People consuming large quantities of citrus fruits, e.g. in California, have more ascorbic acid in their blood than those on a more usual type of diet. A large group of people in England, who appeared healthy and showed no clinical signs of scurvy, had values ranging from 0.3 to 1.3 mg. with an average of 0.65. Only 2 per cent of the cases were below 0.4 mg. (Young, King, Wood and Wootton, 1943, 1946). Prunty and Vass (1943) considered the plasma ascorbic acid a reliable and satisfactory index of the nutritional state with respect to vitamin C. The determination is more easily carried out than a saturation test (cf. urine ascorbic acid). A 'state of saturation' is usually attained when the plasma ascorbic acid is 0.8 mg. per 100 ml. or greater.

Dichlorophenol-Indophenol Method

PRINCIPLE

The oxidation-reduction dye dichlorophenol-indophenol is used to titrate a standard solution of pure ascorbic acid, which is prepared so as to be of about the same concentration as that of a deproteinized filtrate of blood plasma. The volumes of standard solution and of plasma filtrate used to decolorize a standard amount of the dye are then used to calculate the ascorbic acid concentration of the plasma.

METHOD

At least 5 ml. of oxalated blood are required. The plasma should not be separated until just before the preparation of the protein-free filtrate. 2 ml. of plasma are diluted with 8 ml. of 2.5 per cent metaphosphoric (or 5 per cent trichloroacetic) acid, mixed and centrifuged. The clear supernatant

fluid is then run (from a 5 ml. burette*) into 0.05 ml. of dye until all trace of pink colour disappears. The titration should be made quickly so as to minimize the small amount of reduction of the dichlorophenol-indophenol which may take place due to traces of non-ascorbic acid reducing substances in the plasma filtrate. Any froth which might make the titration difficult can be cleared by touching with fine wire which has been dipped in caprylic alcohol.

To 2 ml. of the dilute standard solution are added 8 ml. of 2.5 per cent metaphosphoric (or 5 per cent trichloroacetic) acid. This is titrated against 0.05 ml. of the dye solution, conveniently contained in a conical centrifuge tube. The ascorbic acid solution is run into the dye from a 5 ml. burette until all trace of pink colour has disappeared. The titration gives the ascorbic acid equivalent of 0.05 ml. of dye.

CALCULATION

$$\text{Ascorbic acid}\dagger = \frac{\text{Titration of standard}}{\text{Titration of plasma filtrate}} \times 1.2$$

† mg. per 100 ml. plasma.

SOLUTIONS

Preparation of Dye. 200 mg. of the dye (dichlorophenol-indophenol) are extracted with 50 ml. portions of hot water, and each extract poured through a filter into a 250 ml. volumetric flask. The solution is cooled and diluted to the mark. This stock solution will keep about 2 weeks. For daily use, 10 ml. are diluted to 100 ml. with water which has been freshly boiled and cooled. A method for making the solution from a dichlorophenol-indophenol tablet is given on p. 140.

Preparation of Standard Ascorbic Acid Solution. 60 mg. of ascorbic acid are weighed and dissolved in 100 ml. of 5 per cent acetic acid. 1 ml. of this solution is diluted to 50 ml. with 5 per cent acetic acid. This dilute standard solution contains 1.2 mg. ascorbic acid in 100 ml.

Metaphosphoric Acid. Prepared fresh daily by grinding 2.5 g. HPO_3 with 95 ml. of water until in solution.

Trichloroacetic acid. 5 g. dissolved in water and made to 100 ml.

* Or conveniently, after acquiring a little skill, from a 2 or 5 ml. graduated pipette held in the left hand so that the right is free for shaking the vessel containing the dye solution.

Dinitrophenylhydrazine Method

PRINCIPLE

The 2:4-dinitrophenylhydrazine method of Roe and Kuether (1943) estimates ascorbic acid after oxidation. It is therefore not necessary that the plasma be from a freshly taken specimen of blood as with the dichlorophenol-indophenol method. The plasma is deproteinized with trichloroacetic acid and shaken with activated charcoal in order to oxidize the ascorbic acid to dehydro-ascorbic acid. By treatment with dinitrophenylhydrazine in the presence of thiourea an intensely red-coloured bis-hydrazone is formed which is estimated colorimetrically after treatment with concentrated sulphuric acid.

METHOD

1 ml. of plasma is treated with 4 ml. of 7 per cent trichloroacetic acid and thoroughly shaken. 0.15 g. of activated charcoal are added after 5 minutes and the mixture again shaken well. After 10 minutes the proteins and the charcoal are centrifuged down, and the supernatant passed through a filter paper (7 cm.).

Test. 2 ml. of filtrate, in a colorimeter tube marked at 5 ml., are treated with 0.1 ml. of thiourea solution and 0.5 ml. of dinitrophenylhydrazine. The tube is stoppered with a rubber bung and kept at 37° for 3 hours. The tube is placed in ice-water and 1 ml. of concentrated sulphuric acid is added while the solution is stirred with a glass rod. The colour is allowed to develop for 30 minutes, water added to the 5 ml. mark and mixed.

Standards. 3 standards are prepared corresponding to 0.5, 1.0 and 2.0 mg. ascorbic acid per 100 ml. 5 ml. of the working standard are treated with 0.15 mg. of activated charcoal, in the same way as the plasma and trichloroacetic acid, shaken and filtered after 5 minutes. 0.5 ml., 1 ml. and 2 ml. of working standard ($\equiv$0.002, 0.004 and 0.008 mg. ascorbic acid) are diluted to 2 ml. (in the case of the first two) and then treated with thiourea, dinitrophenylhydrazine and sulphuric acid in the same way as the test.

Blanks. (*a*) *For the Test.* The blank is prepared in the same way as the test, except that the dinitrophenylhydrazine is added after the sulphuric acid.

(*b*) *For the Standard.* 2 ml. of water are used instead of the 2 ml. of ascorbic acid solution ; the other reagents are the same.

The colours of the test and the appropriate standard can be compared in a Duboscq colorimeter ; but it is preferable to read them (test, standards and blanks) in a photoelectric colorimeter, the readings of the appropriate blanks being subtracted from those of the test and the standard. A green 624 light filter should be used. The standard whose colour is most nearly equal to that of the test is used.

CALCULATION

Photoelectric Colorimeter (comparison with e.g. the 1 ml. ascorbic acid working standard).

$$\text{Plasma ascorbic acid*} \begin{cases} = \dfrac{\text{Reading of test} - \text{test blank}}{\text{Reading of standard} - \text{standard blank}} \times 0.004 \times \dfrac{100}{0.4} \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 1 \end{cases}$$

* mg. per 100 ml. of plasma.

Duboscq Colorimeter. The calculation is the same as above, the readings having been made against a grey screen and expressed as extinctions (see p. 197).

SOLUTIONS

Trichloroacetic Acid. 7 g. dissolved in 100 ml., as for sodium, p. 53.

Standard Ascorbic Acid Solution. 50 mg. of ascorbic acid dissolved in 50 ml. of water (≡1 mg. per ml.). *Working Standard.* 0.2 ml. of standard solution diluted to 50 ml. with 7 per cent trichloroacetic acid (≡0.004 mg. ascorbic acid per ml.).

Activated Charcoal. 50 g. of commercial activated charcoal are boiled with 250 ml. of approximately N-hydrochloric acid. The mixture is filtered on a Büchner funnel and the charcoal washed

frequently with water until the filtrate no longer shows a test for chloride or for iron. It is then dried in an air oven at 105° and stored in a wide-necked bottle.

Thiourea. 2.5 g. of thiourea are dissolved in a mixture of 50 ml. ethyl alcohol and 50 ml. of water. This solution should be made fresh each month.

Dinitrophenylhydrazine. 2 g. of 2 : 4-dinitrophenylhydrazine are dissolved in 100 ml. of 10 N-sulphuric acid (27 ml. H_2SO_4 mixed with water, cooled and diluted to 100 ml.) and preserved in a refrigerator.

Sulphuric Acid. Concentrated.

SALICYLATES

It is frequently required to estimate the concentration of salicylate in the plasma of persons receiving aspirin or salts of salicylic acid. This is easily done by the phenol method used for phosphatase (p. 70). 0.2 ml. of plasma, 4 ml. of water and 1.8 ml. of dilute Folin-Ciocalteu reagent are mixed, centrifuged or filtered, and 4 ml. of the filtrate treated with 2 ml. of 15 per cent sodium carbonate. The blue colour is compared with the standard used for phosphatase, and the calculation is similar, i.e. Plasma Salicylates = (Reading of Test/Reading of Standard) × 30,* in mg. per 100 ml. expressed as phenol. By this method normal plasma contains about 4 mg. phenolic substances per 100 ml., and values up to about 20 mg. are found in persons undergoing salicylate therapy.

* For most plasma it is preferable to use a standard one-fifth the strength (≡6 mg.) that of the standard used for phosphatase. This is most easily done by preparing a '*standard-phenol-and-reagent*' one-fifth the strength of that stated on p. 72, i.e. 1 ml. of stock and 100 ml. of dilute Folin-Ciocalteu diluted to 500 ml.

Chapter IV

PROCEDURES FOR SERUM

TAKING OF BLOOD FOR SERUM

About 10 ml. of blood are drawn (as for plasma) and allowed to flow gently into a clean dry vessel. This is then kept at body temperature until the serum has separated. The blood must not be chilled, as this causes hæmolysis as well as abnormal plasma/cell distribution of certain ions. The serum is poured from the clot into a centrifuge tube and is finally centrifuged.

SERUM CALCIUM

There are normally present 9.6 to 10.9 mg. of calcium in 100 ml. (4.8 to 5.45 milli-equiv. per litre) of serum from freshly clotted blood. In hyperparathyroidism and conditions of generalized bone disease this amount may be raised by 2 or 3 mg. and occasionally in severe cases by 5 mg., or even more. In infantile tetany, or in tetany resulting from removal of part or all of the parathyroid glands, the serum calcium is lowered to 7 or 8 mg., and values as low as 6 mg. are sometimes encountered. Those cases of nephritis where an acidosis with raised blood phosphate is present may also show a lowered serum calcium.

PRINCIPLE

The calcium is precipitated from diluted serum by oxalate, and the washed precipitate of calcium oxalate is titrated in acid solution with standard potassium permanganate. From the equation (see standard solutions, p. 188) it can be calculated that 1 ml. of 0.01 N-permanganate is equivalent to 0.2 mg. of calcium.

METHOD

2 ml. of clear serum are added to 2 ml. of water in a clean 15 ml. conical centrifuge tube and 2 ml. of saturated (4 per

cent) ammonium oxalate added, with thorough mixing of the liquids, e.g. by quick rotation between the palms of the hands. After 30 minutes the mixture is centrifuged (10 minutes), and the supernatant fluid carefully poured off so as not to disturb the precipitate. The tube is allowed to drain for several minutes by standing it, top down, on a piece of filter paper, and then wiping the rim clean of adhering fluid. The precipitate is washed by blowing in 3 ml. of dilute ammonia (2 ml. concentrated ammonia diluted to 100 ml. with distilled water), so that the mat of calcium oxalate at the bottom of the tube is thoroughly stirred up. A further ml. of dilute ammonia is used to wash down the sides of the tube which is centrifuged immediately and drained as before. The washing is repeated.

The washed precipitate of calcium oxalate is dissolved in 2 ml. of N-sulphuric acid by warming the tube to 70–80°C. in a beaker of warm water. Titration is carried out by adding N/100 potassium permanganate drop-wise from an accurate 2 ml. burette. (The size of the drops delivered can be diminished by coating the tip of the burette with paraffin wax.) The tube should be kept warm by immersion in the hot water during the titration. The first drop of permanganate to produce a 'persistent' pink colour (for at least 2 minutes) is taken as the end point. A 'blank' titration should be carried out with 2 ml. of N-sulphuric acid (with no calcium) and the amount of permanganate used in the blank (usually 1 drop) subtracted from the former titration.

CALCULATION

$$\text{Calcium}^* = \text{ml. } 0.01 \text{ N-KMnO}_4 \times 0.2 \times \frac{100}{2}$$

$$= \text{ml. } 0.01 \text{ N-KMnO}_4 \times 10$$

* mg. per 100 ml. serum (÷2 = milli-equivalents per litre).

Method for 0.5 ml. of Serum

This procedure depends on the use of a microburette such as that shown in Fig. 6 (see Conway, 1947). One-quarter quantities are used throughout.

0.5 ml. of serum is treated in a 5 ml. conical centrifuge tube with 0.5 ml. of water and 0.5 ml. of ammonium oxalate. The conditions of precipitation, etc., are the same as above: 2 ml. portions of ammonia water being used for the washings. The precipitate is dissolved in 0.5 ml. N-sulphuric acid, and titrated hot with 0.015 N-potassium permanganate while the

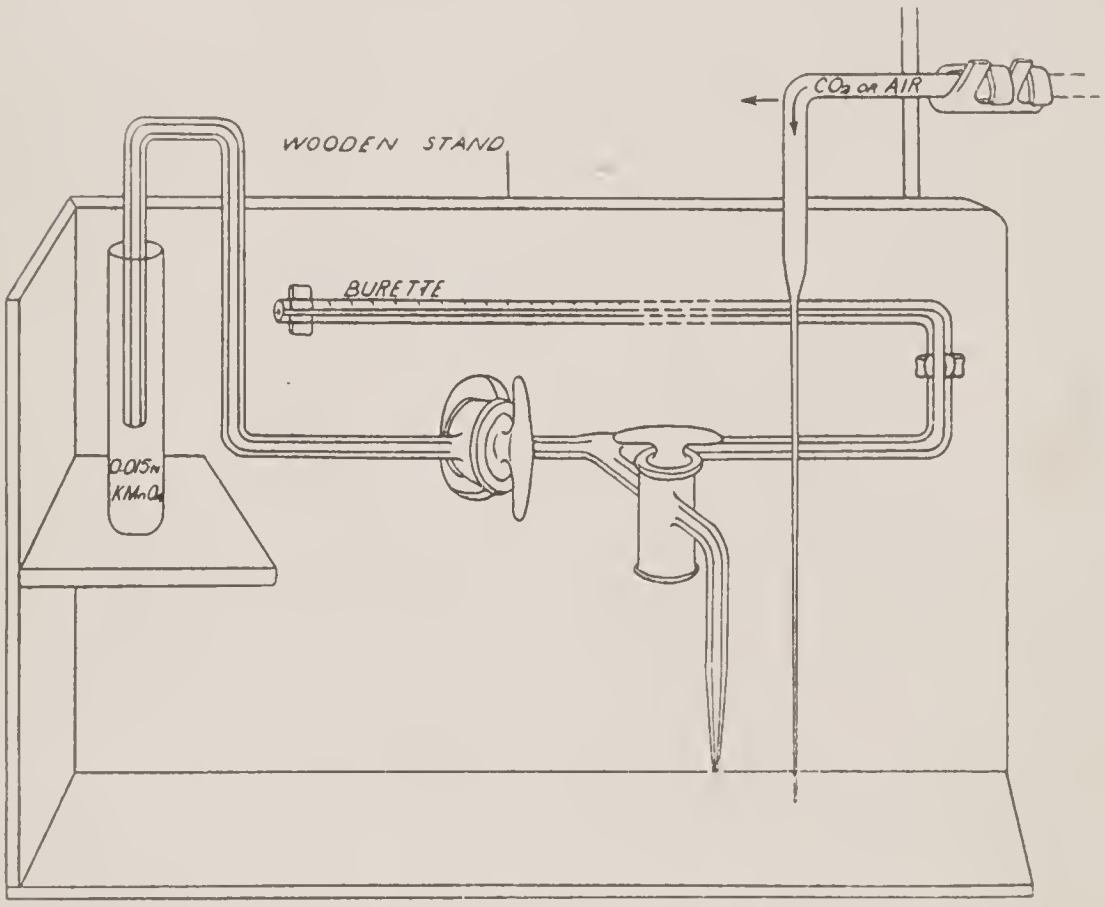


FIG. 6. Conway microburette.

solution is stirred by bubbling air or CO_2 through it from a fine tube. The calculation is:—

Calcium (mg. per 100 ml.) = ml. 0.015 N-$KMnO_4 \times 60$*

*$0.3 \times \frac{100}{0.5} = 60$; (1 ml. 0.015 N-$KMnO_4 \equiv 0.3$ mg. Ca).

SERUM POTASSIUM

The serum of normal persons contains 15–20 mg. of potassium per 100 ml. (4–5 milli-equivalents per litre). This is a

very constant value. There is a rise in Addison's disease, a small rise during attacks of bronchial asthma and in advanced renal disease, and a fall in familial periodic paralysis during the attacks of paralysis, and in treated diabetic coma consequent on a shift of extra-cellular electrolyte into the cells.

PRINCIPLE

The potassium is precipitated as cobaltinitrite. The precipitate is washed and dissolved in hot water. Alkaline solutions of cobalt salts, in presence of a trace of amino-acid (glycine or alanine), reduce the phosphomolybdic-phosphotungstic acid phenol reagent to a blue colour, the intensity of which is directly proportional to the amount of cobalt present, and hence, if potassium has been precipitated as cobaltinitrite, to the amount of potassium in the original solution.

METHOD

Because there are only 15–20 mg. potassium per 100 ml. of serum, and blood corpuscles contain about 300 mg. per 100 ml., there must obviously be no hæmolysis. Also, the serum must be separated from the clot soon after the blood is taken ; otherwise potassium will diffuse out into the serum. Separation of serum 1–2 hours after taking the blood is sufficient.

Test. This method is adapted from the procedures of Jacobs and Hoffman (1931) and Abul-Fadl (1949). 0.2 ml. of serum (and of standard potassium solution) are placed in conical centrifuge tubes graduated at 6 ml., and 0.5 ml. of filtered Kramer and Tisdall's sodium cobaltinitrite reagent is added slowly with constant shaking. After 45 minutes 1 ml. of water is added, and the contents are mixed and centrifuged at moderate speed for 15 minutes. The tubes are then inverted and drained briefly on filter paper ; 2 ml. of water are added down the sides of the tubes without disturbing the precipitate. The tube is again centrifuged for 5 minutes, inverted and thoroughly drained. The precipitates are washed with 5 ml. of 70 per cent alcohol, and centrifuged and drained. The alcohol is blown into the tubes so as to agitate the precipitates.

2 ml. of water are added and the tubes placed in a boiling water-bath until dissolution is complete.

Standard. In another graduated centrifuge tube is placed 1 ml. of the standard cobalt solution (≡0.04 mg. potassium); and 1 ml. of water.

Blank. 2 ml. of water.

To each of the tubes, while hot, 1 ml. glycine solution and 2 ml. Na_2CO_3 solution are added and thoroughly mixed. 1 ml. diluted Folin-Ciocalteu phenol reagent is then added to each, the contents are mixed again, and the tubes are allowed to stand in a water bath at 37°C. for 10–15 min. After cooling to room temperature the volume is accurately adjusted to 6 ml. in each tube, and the colours are read in a colorimeter, using a red filter 608 (p. 203). The colours are stable for several hours.

CALCULATION

Photoelectric Colorimeter.

$$\text{Serum potassium}^* \begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} = 0.04 \times \dfrac{100}{0.2} \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 20 \end{cases}$$

* mg. per 100 ml. serum (÷3.9=milli-equivalents per litre).

Duboscq Colorimeter. The calculation is the same as above, except that the ratio of readings is inverted to Reading of standard/Reading of test (see p. 196).

SOLUTIONS

Sodium Cobaltinitrite Reagent (Kramer and Tisdall, 1921). Solution A: 25 g. of cobaltous nitrate crystals are dissolved in 50 ml. of water, and to this solution are added 12.5 ml. of glacial acetic acid. Solution B: 120 g. of sodium nitrite (potassium free) are dissolved in 180 ml. of water, giving a total volume of about 220 ml. To the whole of A is added 210 ml. of B. Nitric oxide is evolved. Air is drawn through the solution till all the gas has passed off. The reagent thus prepared is best kept in the ice-chest and should be filtered each time before use.

Standard Potassium Solution. 0.2228 g. of potassium sulphate (K_2SO_4) is dissolved in 500 ml. of water, giving a solution equivalent to 0.04 mg. K per 0.2 ml. (20 mg. potassium per 100 ml.).

Standard Cobalt Solution (≡0.04 mg. potassium per ml.). 202.4 mg. of cobalt ammonium sulphate is dissolved in a litre of water. Any cobalt salt may be used but this is the easiest to weigh. The solution should be standardized by the above method against the standard potassium solution, and may thereafter be used in place of the latter, with only an occasional check of its strength.

Glycine (1M). 7.5 g./100 ml. water. The solution is filtered and preserved with a few drops of chloroform.

Sodium Carbonate Solution (15 per cent w/v). Anhydrous Na_2CO_3 (15 g.) is dissolved in warm water and made to 100 ml. This solution is kept in a warm place.

Phenol Reagent of Folin and Ciocalteu (1927). For method of preparation see p. 72. This reagent is diluted for use (1 vol. reagent+2 vols. water).

THIOCYANATE

Determinations of the concentration of thiocyanate in the serum (or plasma) are useful as an estimation of the total extracellular fluid of the body.

PRINCIPLE

Thiocyanate is estimated by its colour reaction with ferric nitrate and comparison with a standard. Bowler (1944) has shown that ferric nitrate must be in large excess, and the final solution at least 0.5 N with respect to nitric acid in order to rule out interfering substances, and to produce a colour which obeys Beer's law.

METHOD

To 0.5 ml. of serum (or plasma) are added 4.5 ml. of 5 per cent trichloroacetic acid. The mixture is filtered through a No. 40 Whatman filter paper.

Test and Standard. To 3 ml. of filtrate (≡0.3 ml. serum) and 3 ml. of standard thiocyanate are added 3 ml. of ferric nitrate reagent; and the coloured solutions mixed in the absence of daylight.

The colour densities are measured within 5 minutes, using a blue (622) light filter.

CALCULATION

Photoelectric Colorimeter.

$$\text{Thiocyanate}^* \begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.029 \times 3 \times \dfrac{100}{0.3} \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 29 \end{cases}$$

* in mg. of CNS ion, per 100 ml. of serum.

SOLUTIONS

5 per cent Trichloroacetic Acid.

Ferric Nitrate. 80 g. $Fe(NO_3)_3.9H_2O$ dissolved in 250 ml. 2 N-nitric acid (p. 190), made to 500 ml. with water and filtered.

Stock N/10 *Potassium Thiocyanate.* This should be tested against silver nitrate (p. 135).

Standard Thiocyanate. N/2000. 0.5 ml. of stock diluted to 100 ml. (1 ml. ≡ 0.0485 KCNS, or 0.0405 NaCNS, or 0.029 CNS.)

SERUM COLLOIDAL GOLD REACTION

(Gray, 1940; MacLagan, 1946)

A stable colloidal gold solution is prepared in a defined buffer, and mixed with a small amount of serum. Precipitation and flocculation occur in various degree, according to the nature of the serum, in a manner rather similar to the Lange colloidal gold reaction with C.S.F. Negative results are usually obtained in obstructive jaundice and positive results, depending in degree on the severity of the disease, in cirrhosis and infective hepatitis.

METHOD

0.5 ml. of serum is added to 0.5 ml. of buffer, and 2.5 ml. of colloidal gold solution are added. The mixture is allowed to stand overnight.

RESULTS

The results are expressed as numbers, corresponding to the degree of turbidity or precipitation produced.

Complete precipitation	=5
Partial precipitation, supernatant just coloured .	=4
Gold precipitate visible only on pouring out the solution from the test tube, change in colour .	=3
Obvious change in colour, and slight turbidity .	=2
Slight change in colour, and very slight turbidity	=1
No change in colour	=0

SOLUTIONS

Colloidal Gold Solution. To 100 ml. of distilled water are added 1 ml. of 1 per cent potassium oxalate and 0.4 ml. N/50 sodium hydroxide (carbon dioxide free). The mixture is heated to 60–70°C. and 1 ml. of 1 per cent sodium gold chloride is added drop by drop with mixing until the colour changes to a ruby red.

Buffer Solution, pH 7.8. 0.55 g. barbitone, 0.412 g. sodium barbitone and 0.2 g. phenol are dissolved in 100 ml. of distilled water with warming.

THYMOL TURBIDITY TEST

(MacLagan, 1944, 1947)

When a small amount of serum from a normal person is added to a buffered solution of thymol the solution remains clear, or at the most, only a very small degree of turbidity develops. Sera from persons suffering from certain clinical conditions, on the other hand, produce variable degrees of turbidity. This is noticeably so in jaundice and particularly in hepatitis, where marked turbidities are produced. The test has been successfully used by MacLagan as an additional means for assisting in the diagnosis of jaundice, and distinguishing hepatitis from obstructive jaundice.

METHOD

0.05 ml. of serum is added to 3 ml. of thymol buffer (a 1:60 dilution), mixed and allowed to stand for 30 minutes to 1 hour. The turbidity is read against the albumin standards used for urine and C.S.F. (p. 97). If the turbidity exceeds that of the 100 mg. standard the mixture is diluted with 3 ml. (or multiples of 3 ml.) of thymol buffer until the turbidity is such that it can be read on the scale.

CALCULATION

The result is in arbitrary units.

$$\text{Thymol turbidity (units)} = \left(\frac{\text{Final dilution}}{60}\right) \times \left(\frac{X \text{ mg. albumin}^*}{10}\right)$$

* The mg. albumin represented by the standard tube which is matched by the thymol turbidity.

Example. Since the standard dilution is 1:60, therefore, if the final dilution is 1:120 and the match is with the 70 mg. standard, the result is 14 units.

The normal limits are 0—4 units.

SOLUTIONS

Thymol Buffer. To 500 ml. of distilled water are added 1.38 g. of barbitone, 1.03 g. sodium barbitone and approx. 3 g. of thymol. The mixture is heated just to boiling, stirred well and then cooled thoroughly. The solution is now turbid, and should be seeded with a small amount of powdered thymol crystals. This solution is allowed to stand overnight at room temperature. It is again shaken well (to avoid super-saturation) and filtered from the crystalline deposit of thymol.

BROMSULPHTHALEIN TEST

PRINCIPLE

The bromsulphthalein test is perhaps the most sensitive and easily executed of the liver function investigations. It is useful, particularly, in the study of mild liver dysfunction and is usually used in the study of the non-jaundiced subject. An amount of the dye is injected and its rate of disappearance from the blood is followed by means of colorimetric estimations in the serum. In normal persons not more than 5 per cent of the dye remains after 30 minutes. In pathological sera more than 10 per cent, and up to 100 per cent of the bromsulphthalein remains. Since this is a very sensitive test of liver insufficiency it is found that retention of the dye occurs in obstructive jaundice as well as in non-obstructive. It is useless, therefore, for the differentiation of jaundice, and

should only be used in an attempt to determine if there is hepatic insufficiency or dysfunction.

METHOD

The dye is a white powder, dissolves easily in water and forms a colourless solution which turns red on the addition of alkali. It can be sterilized by steaming or autoclaving. An amount of the solution containing 2 mg. of bromsulphthalein per kilogram of body weight of the patient is injected into an arm vein. Blood (10 ml.) is taken from the other arm at 30 minutes.

Test. The dye is estimated in the serum obtained from the clotted sample after centrifuging. 2 ml. samples of the sera are treated, one with 2 ml. of N/10-sodium hydroxide, and the other with 2 ml. of N/10-hydrochloric acid to serve as a blank. The optical density of the bright red colour is determined in any photometer (green 624 light filter) or colorimeter; and is compared with that of the 100 per cent standard described below.

CALCULATION

Photoelectric Colorimeter.

$$\text{Retention}^* = \frac{\text{Reading of test}}{\text{Reading of standard}} \times 100$$

* as percentage of injected dye.

The standard may be diluted with water, if necessary, in order to reduce its colour for comparison with that of the 30-minute sample, the necessary factor being introduced into the equation.

Duboscq Colorimeter. The calculation is the same as above, except that the ratio of readings is inverted to Reading of standard/Reading of test (see p. 196).

SOLUTIONS

Bromsulphthalein (sterile). 4 g. of bromsulphthalein are dissolved in 100 ml. (≡40 mg./ml.) and sterilized in the autoclave or by steaming. It is convenient to distribute the solution in 3 ml. sterile ampoules. Since the standard injection is 2 mg. per

kg., 1 ml. of this solution should be used for each 20 kg. of body weight. This corresponds, on the basis of the usual relation of volume of blood plasma to body weight, to about 4 mg. bromsulphthalein per 100 ml. of plasma.

Standard Solution (4 mg. bromsulphthalein per 100 ml). 1 ml. of the bromsulphthalein (sterile) is accurately measured into a litre volumetric flask and made up to volume with water. The *working standard* consists of 2 ml. of this standard solution diluted, like the 2 ml. of blood serum for the test, with 2 ml. of N/10 sodium hydroxide.

ACETONE BODIES

Dumm and Shipley (1946) have described a convenient dilution method for the approximate estimation of aceto-acetic acid in blood serum. This is a useful estimation in the study of diabetes ; and when it is desired to obtain a measure of the total acidic ions of the serum it may be necessary to include the keto acid which may be an appreciable fraction of the total anions. With the method described a minimal or negligible ketonæmia never exceeds 20 mg. per 100 ml. ; in diabetic acidosis more than 50 mg. per 100 ml. is almost invariably found.

PRINCIPLE

A definite positive nitroprusside test is given by a solution containing 10 mg. of ketoacid per 100 ml. At this concentration a violet coloration is produced under the specified conditions, and greater dilutions give a negative test. This makes it possible to gain an approximate but useful measure of the concentration of aceto-acetic acid.

METHOD

Dilutions of the serum with distilled water are tested on a filter paper or (preferably) a white tile. A 'knife-point' of the mixed nitroprusside, ammonium sulphate and sodium carbonate (see below) is treated with a drop of the serum, 1 ml. of which has been diluted with 1 ml. of water. If this gives a positive test, a further 1 ml. of water is added (making a dilution of 1 in 3) to the diluted serum, mixed and another test is made. Repeated further dilutions of the diluted serum

are made with more water until the test is just positive. Although 1 drop is withdrawn from the mixture for each test this does not influence the dilution sufficiently to create an appreciable error in the final result.

CALCULATION

Serum keto acid* = the dilution $\times 10$

* in mg. per 100 ml.

REAGENT

Sodium Nitroprusside, 1 g. very finely ground; *ammonium sulphate*, 20 g.; *sodium carbonate*, 20 g. Na_2CO_3. These are mixed completely, but not ground. The mixture should be kept dry in a glass-stoppered bottle where it will remain stable for about a year. The mixture may also be used to measure the acetone bodies in urine.

Chapter V

PROCEDURES FOR CEREBRO-SPINAL FLUID

The chemical determinations most frequently of value in the examination of cerebro-spinal fluid are those of protein, chlorides, sugar, calcium and urea. Lange's colloidal gold reaction is also of importance.

Globulin tests in normal fluid are negative. Total protein may be increased in many pathological conditions. Chlorides are lowered characteristically in meningitis, especially tuberculous meningitis. Sugar is also lowered in meningitis. Calcium may be lowered in tetany, while the urea value closely parallels the level of blood urea.

Table 7

Composition of C.S.F.

Constituent	Normal range (per 100 ml.)	Clinical conditions in which high values (unless otherwise stated) are found
Urea . . .	15–30 mg.	Increased in nitrogen retention.
Creatinine . .	0.7–1.5 mg.	,, ,, ,, ,,
Sugar . . .	60–100 mg.	Diabetes. Reduced in acute suppurative meningitis.
Chlorides (as NaCl)	700–740 mg.	Nephritis. Decreased in meningitis, particularly tuberculous meningitis.
Proteins (total) .	20–40 mg.	Meningitis. Syphilitic conditions. Froin's Syndrome.
Globulin { Pandy .	reactions	
Globulin { Nonne-Apelt	negative.	—
CO_2-combining power	55–65 ml.	

PRINCIPLES OF METHODS

Total protein is determined by the sulphosalicylic acid method, with the use of permanent standards. The reactions of Nonne-Apelt and Pandy are used to test for globulin. Nonne-Apelt's test depends on the precipitation of globulin by

half-saturation with ammonium sulphate. Pandy's reagent is a saturated aqueous solution of phenol.

For chlorides, Mohr's method is employed. This depends on the titration of the fluid with silver nitrate, until all the chloride is precipitated as silver chloride. The first excess of silver nitrate gives, with potassium chromate added as indicator, a reddish-brown precipitate of silver chromate.

Urea, creatinine, sugar, CO_2-combining power and calcium are estimated exactly as in the case of blood.

METHODS

TOTAL PROTEIN

1 ml. of fluid in a test tube is treated with 3 ml. of aqueous sulphosalicylic acid. After 5 minutes the turbidity is compared against the permanent standards described below. For values above 90 mg. per 100 ml., the fluid must be suitably diluted with water before treatment.

GLOBULIN

(1) *Nonne-Apelt's Test.* 1 ml. of saturated ammonium sulphate in a small tube is carefully layered with about 1 ml. of cerebro-spinal fluid. A white ring at the junction of the liquids indicates globulin.

(2) *Pandy's Test.* 1 drop of fluid is added to 0.5 ml. of Pandy's reagent. A turbidity or precipitate indicates globulin (Pandy, 1910).

SOLUTIONS AND STANDARDS

Sulphosalicylic Acid. 3 g. dissolved in water and diluted to 100 ml.

Ammonium Sulphate. Saturated solution.

Pandy's Reagent. 7 g. phenol dissolved in 100 ml. of distilled water.

Protein Standards. The principle of the method consists in comparing the turbidity in a set of permanent standards with that produced when a standard amount of sulphosalicylic acid is added to a measured amount of the albuminous fluid. The standards consist of a permanent suspension of formazin in gelatin. They have been standardized by reference to the turbidity produced with sulphosalicylic acid in a solution of crystalline

horse-serum albumin, a diluted human serum whose protein content was established by Kjeldahl, and a solution of crystalline albumin isolated from the urine of an orthostatic albuminurie.

The gelatin is clarified as follows: 65 g. of pure gelatin are dissolved at about 90°C. in 500 ml. of water. The 'white' of one egg, in approximately twice its volume of water, is vigorously stirred into the solution. The mixture is heated, with continual stirring, on a boiling water bath for 1 hour. It is then filtered through a large coarse paper in a heated funnel. The clear slightly yellow filtrate is kept liquid at about 50°C. for the preparation of the standards described below.

The formazin is prepared as follows: 25 ml. of an aqueous solution (10 g. per 100 ml.) of hexamine (hexamethylene tetramine) are added to 25 ml. of a solution (1 g. per 100 ml. in water) of hydrazine sulphate. The mixture is stoppered, shaken, and left at room temperature for at least 15 hours. The resulting precipitate of formazin is carefully mixed by gently shaking until it is evenly dispersed throughout the liquid.

14.5 ml. of the suspension are added to 100 ml. of gelatin together with 0.3 ml. of 40 per cent formaldehyde to ensure permanent 'setting.' Such a gelatin suspension of formazin has been found to be equivalent to a concentration of serum albumin (precipitated with sulphosalicylic acid under the conditions described above) of 100 mg. per 100 ml. The gelatin suspension is now diluted with clarified gelatin (containing 0.3 ml. of 40 per cent formaldehyde per 100 ml.) to give standards corresponding to other albumin concentrations. The following mixtures of gelatin and formazin-gelatin suspension are made in small tubes of uniform bore (7.5 by 1 cm.).

Tube	ml. of gelatin	ml. of gelatin-formazin	Value in mg. of albumin per 100 ml.
1	3.6	0.4	10
2	3.2	0.8	20
3	2.8	1.2	30
4	2.4	1.6	40
5	2.0	2.0	50
6	1.6	2.4	60
7	1.2	2.8	70
8	0.8	3.2	80
9	0.4	3.6	90
10	0	4.0	100

When cold, the tubes are stoppered with corks cut level with the top of the tube. The stoppered ends are then dipped in molten paraffin wax and allowed to cool. The standards may be checked

against serum solutions standardized by nitrogen determinations. They should be mounted in a wooden rack painted black. Comparison is best made by viewing them against a strip of white cardboard with a transverse black line fastened to the rack.

Arrangements have been made with Messrs. Gallenkamp and Co., London, to manufacture these standards. Sample sets have been checked against those prepared and against albumin solutions of known strength.

CHLORIDES

0.2 ml. of cerebro-spinal fluid is treated with 1 ml. of water and a drop of potassium chromate solution (5 g. per 100 ml.). The mixture is titrated with M/58.5 silver nitrate solution until a permanent reddish-brown colour is produced. An accurate 2 ml. burette is used.

M/58.5 *Silver Nitrate* contains 2.906 g. of silver nitrate crystals in 1 litre of solution in distilled water. The silver nitrate solution should be kept in the dark in a brown bottle and should be frequently checked against a standard sodium chloride solution (500 mg. per 100 ml.).

CALCULATION

1 ml. of $\frac{M}{58.5}$ silver nitrate $\equiv$ 1 mg. of sodium chloride.

Hence,

$$\text{C.S.F. chloride}^* \begin{cases} = \text{ml. of silver nitrate used} \times \frac{100}{0.2} \\ = \text{ml. of silver nitrate used} \times 500 \end{cases}$$

* mg. NaCl per 100 ml.

The silver iodate and mercuric nitrate methods (p. 54) and the thiocyanate method (p. 133) may also be used for C.S.F. chloride.

LANGE'S COLLOIDAL GOLD REACTION

PRINCIPLE

This reaction depends on the fact that although normal cerebro-spinal fluid has no action on a particular colloidal gold solution, fluid from cases of syphilis, disseminated

sclerosis, or meningitis may cause various degrees of precipitation of the gold at different dilutions of the C.S.F., which are fairly characteristic for each disease.

Typical responses are:—

Luetic . . .	0 1 3 4 3 2 1 0 0 0
Paretic . . .	5 5 5 4 3 2 1 0 0 0
Meningitic . .	0 0 1 1 2 3 2 2 1 0

These figures serve to indicate the degrees of precipitation in tubes 1–10 in that order.

Complete precipitation (clear, colourless supernatant fluid) . . .	is called 5
Partial precipitation (slightly cloudy, light blue supernatant fluid) .	,, ,, 4
Deep blue colour . . .	,, ,, 3
Lilac to purple colour . . .	,, ,, 2
Lilac colour	,, ,, 1
Unchanged red colour . . .	,, ,, 0

A meningitic type of curve is found in all forms of coceal meningitis and tuberculous meningitis. A paretic type of curve is found in general paralysis of the insane (G.P.I.), in tabes, in disseminated sclerosis and rarely in encephalitis lethargica. When a paretic curve occurs in association with a positive Wasserman reaction (W.R.) and is unaffected by antispecific treatment it is symptomatic of G.P.I. rather than tabes: when it occurs with a negative W.R. it is strongly suggestive of disseminated sclerosis. A luetic type of curve occurs in all forms of cerebral syphilis. It may also be found in disseminated sclerosis and is more common in encephalitis lethargica than the paretic type.

METHOD

It is essential that all glassware used be perfectly clean, and that, for all solutions, water twice distilled from glass should be used. A chromic acid mixture (20 g. of potassium dichromate dissolved in a minimum amount of water and the volume made to 1 litre with concentrated sulphuric acid) may

be used for cleaning glass apparatus, which should be stored in the mixture when not in use.

Ten small perfectly clean test tubes (1.0×7.5 cm.) are employed. In the first tube is placed 0.9 ml. of sodium chloride solution (0.4 g. per 100 ml. in re-distilled water); and 0.5 ml. of the chloride solution is placed in each of the other nine tubes. To the first tube is added 0.1 ml. of the cerebro-spinal fluid. 0.5 ml. of the mixed contents of this tube are placed in tube 2; 0.5 ml. of this mixture from 2 is transferred to tube 3, and so on, until tube 10 is reached; from this 0.5 ml. is discarded. The dilutions of fluid are then 1 : 10; 1 : 20; 1 : 40; ....1 : 5120. 2.5 ml. of colloidal gold (see below) are added to each tube, and the stoppered tubes left for 16–24 hours. At the end of this time precipitation is gauged as indicated above.

SOLUTION

Colloidal Gold Solution. (*a*) *Oxalate Method.* About 50 ml. of re-distilled water are heated rapidly to boiling in a hard glass flask. This water is discarded and the flask is washed out with fresh cold re-distilled water. 100 ml. of the water and 1 ml. of filtered 1 per cent potassium oxalate solution are then heated in the flask almost to boiling point. The gold chloride solution (1 g. $NaAuCl_4.3H_2O$ per 100 ml.) is then added drop by drop from a 1 ml. pipette to the continuously agitated contents of the flask until the mixture assumes a bright red colour, when it is removed from the flame. The rest of the 1 ml. of gold solution is then added more rapidly. The colloidal gold so formed should be bright cherry-red in colour and should show practically no cloudiness or fluorescence. It will keep for a considerable period. This preparation requires considerable practice.

(*b*) *Citrate Method.* In a litre flask are placed 675 ml. water twice distilled from glass. The flask is closed with a ground-in reflux condenser and brought to the boil. 7 ml. of 1 per cent gold chloride solution are added, followed by 17.5 ml. of 1 per cent trisodium citrate solution. The boiling is continued for 15 min. The flame is removed and the mixture allowed to cool. The resulting solution is a clear sparkling red colour.

To standardize this solution, four 50 ml. samples are taken and to these in turn are added 0.1, 0.15, 0.175, 0.2 and 0.225 ml. of N/10 hydrochloric acid. These are put up against a normal and a known paretic C.S.F. The mixture is selected which gives the strongest reaction with a paretic fluid, yet does not give a reaction

greater than 0110000000 with a normal fluid. The corresponding quantity of acid is added to the main bulk of solution, thoroughly agitating the mixture during the addition.

If a paretic C.S.F. is not available, a solution of 0.8 per cent washed hæmolysed sheep cells, used in the test like a C.S.F., will give a paretic curve.

(*c*) *Citrate-Hydrogen Peroxide Method.* 92 ml. of the twice distilled water are heated to the boil, the flame removed, and 1 ml. of the gold chloride solution (1 g. $NaAuCl_4.3H_2O$ per 100 ml.) added with thorough mixing. 5 ml. of 1 per cent trisodium citrate are added, with shaking, to the still hot mixture, and finally 2 ml. of water to which 0.02 ml. of hydrogen peroxide solution has been added.

Chapter VI

PROCEDURES FOR FÆCES

BLOOD IN FÆCES

The recognition of small amounts of blood in fæces ('occult blood') indicates the presence of a hæmorrhage somewhere in the intestinal tract. Bleeding from the mouth and throat, and contamination of the fæces by menstrual or hæmorrhoidal bloods should, of course, be ruled out. (In the latter case the undecomposed blood may be detected macroscopically—usually as superficial patches on the stool.)

'Occult blood' occurs fairly frequently in gastro-intestinal cancer and in gastric and duodenal ulcer. Its detection is therefore of considerable value as an aid to diagnosis. Repeated tests are advisable as bleeding may be intermittent and a single negative result is not necessarily significant. A single positive test, or a series of positive tests, on a properly controlled patient, will usually be found to be significant of the presence of some type of ulcer.

Certain of the degradation products of hæmoglobin formed during the digestion of meat will give a positive test if they are present in the fæces. Chlorophyll has been alleged to do likewise. It is consequently essential to maintain the patient on a meat-free and green vegetable-free diet for at least three days before making the examination.

PRINCIPLE

The chemical tests for blood depend on the fact that the active grouping involving iron in hæmoglobin transfers oxygen from hydrogen peroxide to certain oxidizable substances (benzidine, *ortho*tolidine, gum guaiacum and pyramidone are the most common) to give coloured substances.

Benzidine Reaction

(*a*) A thin fæcal suspension is made by shaking a small amount of fæces (about as big as a pea) with 5 ml. of water in a test tube. The mixture is boiled to inactivate enzymes. (These, as well as blood, may give positive results, but unlike the active grouping of hæmoglobin, they are destroyed by heat.) The extract is cooled ; about 1 ml. of it, in another test tube, is treated with 2 ml. of benzidine solution and 1 ml. of hydrogen peroxide solution. If the test is positive a blue colour develops at once.

(*b*) A little fæces is smeared on a glass slide, which is then placed on a boiling water-bath for 3 minutes. A mixture of benzidine solution and hydrogen peroxide solution (2 : 1) is dropped on the smear. A blue colour is indicative of a positive test.

Gum Guaiacum Reaction

1 ml. of a boiled and cooled suspension of fæces, made as above, is mixed in a test tube with 1 ml. of an alcoholic solution of guaiacum resin. 1 ml. of hydrogen peroxide is then added and the tube is well shaken, with further additions of alcohol to dissolve the precipitated resin. A blue colour indicates a positive test. This test may be modified, as above, for use with slides : in this case a clear mixture (approximately 2 : 1) of alcoholic resin and hydrogen peroxide is dropped on the dried fæcal smear.

SOLUTIONS

Benzidine Solution. Approximately 3 g. per 100 ml. made by shaking 3 g. of benzidine in cold glacial acetic acid (100 ml.) until solution is complete. 'Analytical Reagent' (A.R.) benzidine should be used.

Hydrogen Peroxide Solution. '10 vols.' (3 per cent).

Gum Guaiacum in Alcohol ('Tincture of Guaiac'). Approximately 1 g. of resin is dissolved in 100 ml. of industrial spirit. The solution will keep for about a month.

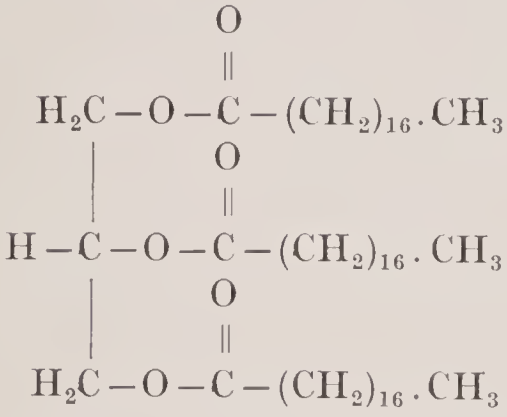
FAT IN FÆCES

Normal fæces contain about 10–25 per cent of mixed fats (dry weight). These consist of a small amount of neutral fat.

1–5 per cent; free fatty acids, 5–13 per cent; and fatty acids present as soap (e.g. sodium salt of fatty acid), 5–15 per cent.

The following are representative formulæ:—

Neutral Fat.



Tristearin
(glyceryl tristearate)

Free Fatty Acid. Stearic acid: $CH_3.(CH_2)_{16}.CO_2H$.

Soap. Sodium stearate: $CH_3.(CH_2)_{16}CO_2Na$.

In clinical conditions in which there is either deficiency of digestion or of absorption, the amount of total fat may be markedly increased. Thus in pancreatic insufficiency the total fat may constitute 60–80 per cent of the dry weight of the fæces. Most of the increase is in the neutral fat and may be due to insufficient pancreatic secretion. There is also an increase of fat in cases of deficient bile secretion such as obstructive jaundice. The increase here is mainly due to fatty acid. The hydrolysis of the fats is normal, but they are improperly absorbed, apparently due to a lack of sufficient bile to bring about proper emulsification.

In normal children the fæcal fat is higher (average about 30 per cent) than it is in adults; and may, in infants, be 50 per cent (or occasionally even more). About one third is present as neutral fat.

PRINCIPLE

The fats are extracted from the dried fæces by means of an anhydrous solvent (ether) and the weight of the material extracted is ascertained by weighing the residue obtained

when the extraet is evaporated to dryness. The neutral fat and the free fatty aeid of the fæces are solublc in ether and can be extracted directly from the fæces, but it is first neeessary to release thc fatty acids eombined as soap by treatmcnt with hydrochloric aeid, before they ean be extraeted. Free fatty aeid is determined by alkali titration.

METHOD

The fæces are weighed, thoroughly mixed and prepared for analysis by drying a weighed samplc in a porcelain dish on a hot water bath, and then transferring to a vaeuum desieeator over ealeium chloride. The dry speeimen is weighed, carefully powdered and thoroughly mixed in a mortar.

A. Total Fat. The fæces are treated with hydrochloric aeid to liberate fatty aeid present as soap and the whole of the fats extraeted with ether. 0.5 g. of the fæees is transferred to a 100 ml. pyrex glass stoppered cylinder. 10 ml. of water and 3 ml. cone. hydroehloric acid are added. The cylinder is plaeed in a hot water bath for 10 minutes.* After cooling to room temperature 50 ml. of ether are added and thc eontents thoroughly mixed by inverting thc tube 60–80 times. The glass stopper should be loosened 2 or 3 times during the shaking to relieve the pressure of the ether vapour. By this means all thc fat of the fæees should pass into the ether. The cylinder is left stoppered in a vertieal position until the ether layer is completely separated. 25 ml. of the ether extraet are now removed and are plaeed in a weighed evaporating basin. The ether is removed by warming thc dish on a water-bath or a hot plate and then plaeing in a vaeuum desiceator containing a few lumps of paraffin wax in a beaker. The inerease in weight in the dish represents the fat content of half the total ether extraet and hence of 0.25 g. of dried fæees. The result is expressed as g. total fat per 100 g. dried fæces.

B. Neutral Fat plus Free Fatty Acids (Unsoaped Fat). An ether extraction of thc fæees which have not been treated with hydrochlorie aeid will dissolve out the neutral fat and

* Care must be taken to warm and cool thc cylinder gradually, otherwise it will crack.

the free fatty acid without removing any of the fat present as soap. 0.5 g. of dried fæces is transferred to another 100 ml. glass-stoppered cylinder and 10 ml. of water are added without the hydrochloric acid used in the case of total fat. After warming for 10 minutes as in 'A' the cylinder is cooled and 50 ml. of ether added. The ether-soluble fat is extracted as before by thorough mixing of the contents of the tube. 25 ml. of the extract are removed to a weighed dish and from the weight of the residue the weight of neutral fat plus free fatty acid in 0.25 g. of dried fæces is obtained.

C. Free Fatty Acid. The free fatty acids in the residue from (B) are determined by titration with N/10 sodium hydroxide in alcohol. The residue is dissolved in 10–20 ml. of absolute alcohol, stirring with a glass rod and warming if necessary, and a few drops of phenolphthalein added. The alcoholic sodium hydroxide is run in from a burette (2 ml.) until the production of the first permanent pink colour. The result is calculated on the arbitrary assumption that all the free fatty acid present is stearic acid (molecular weight 284).

1 ml. N/10 sodium hydroxide≡0.0284 g. free stearic acid.

D. Fatty Acid Present as Soap. By subtracting the weight of the residue of the ether extract of neutral fat and fatty acids from that of the extract of total fat the weight of the fatty acid present as soap may be obtained, i.e. D = A − B.

E. Neutral Fat. By subtracting the amount of free fatty acid (obtained by titration) from the weight of (B) (the neutral fat plus free fatty acid) the amount of neutral fat can be obtained, i.e. E = B − C.

SOLUTION

N/10 *Sodium Hydroxide in Alcohol.* 10 ml. of N-sodium hydroxide in water are diluted to 100 ml. with absolute alcohol. This solution turns brown on keeping, but this does not affect the titration appreciably.

FAT BALANCE TEST

In normal people, the percentage of fat absorption is 95 per cent or more on a 100 g. fat intake, and 90 per cent may be taken as the demarcation between abnormal results, and

normal or equivocal results. A result of less than 90 per cent indicates steatorrhœa, but does not, of course, determine the ætiology of the fat absorption defect. In patients with severe steatorrhœa, even a fat intake of 100 g. per day may be clinically harmful. In such cases, the examination of a random stool specimen should suffice to confirm the diagnosis, by demonstrating a fat content of over 30 per cent of the dried fæces. If this investigation gives a doubtful result, a 100 g. per day fat diet can safely be given, and the fat balance assessed by the criteria given above. A fat balance test is also useful when steatorrhœa is not clinically obvious, but is being considered as a possible cause of anæmia, osteoporosis, chronic diarrhœa, or malnutrition.

PRINCIPLE

This procedure is designed to increase the precision with which steatorrhœa can be detected. In patients with minimal steatorrhœa, a single sample of stool may have a fat content within the accepted normal limits. This may happen because of random variation in the disease process or in stool collection, or because the stool in question was derived from a meal or meals in which little fat was consumed. In a fat balance, the patient is given a diet containing a known amount of fat, in the range of 80–120 g. per day ; and the stools for a 3-day period are collected and analysed for their total fat content. The difference between dietary fat and fæcal fat is taken as a measure of absorbed fat. This involves a systematic error, in that some of the fæcal fat is excreted by the intestine, and does not represent unabsorbed fat ; but error from this cause is slight so long as the fat content of the diet is fairly high, so that truly excreted fat forms only a small proportion of the fæcal fat.

METHOD

The dietary control required over a period of at least 3 days makes it necessary to admit the patient to hospital. There he is given a diet of constant daily composition, with 80–120 g. fat per day. The most satisfactory way of doing this is to supply the fat in a few articles of the diet which can be

carefully weighed, and make up the rest of the diet with foods of very low fat content. For example, 100 g. of fat can be supplied by the following foods :—

Article of food	Amount (oz.)	Fat content (g.)
1 Egg	2	7
Milk	20	22
Butter	$2\frac{1}{2}$	60
Steamed white fish (halibut, plaice, cod or sole) . .	4	3
Lean meat	2	8
		100

The remainder of the diet is made up of bread, potato, green vegetables, sugar, preserves, fruit and cereals other than oatmeal. Capsules containing 0.5 g. of carmine are given with the first meal of the diet, and the first carmine-coloured stool is saved, together with subsequent stools. After 3 days on the diet, the patient is given 0.5 g. carmine with his next meal and the 3-day specimen ends with the last uncoloured stool. The accuracy of the test is increased by giving the constant fat diet for 2 days before the initial carmine marker is given, and it may sometimes be necessary to use a longer collection period than 3 days in those patients whose stools remain tinged with carmine for longer than usual. The total 3-day stool is thoroughly mixed and weighed. A sample is taken for the determination of total fat, as previously described. If the fæcal fat be A, and the dietary intake of fat over the collection period be F, then the percentage fat absorption is $\frac{F-A}{F} \times 100$ (method of Black, 1950).

UROBILINOGEN

The normal range of fæcal urobilinogen is 30–220 mg. per 100 g. of dried fæces ; and the normal daily output, 22–120 mg. (MacLagen, 1946).

PRINCIPLE

The stercobilin pigments of the fæces are reduced to urobilinogen. This is extracted with water, and the solution treated with Ehrlich's dimethylaminobenzaldehyde reagent

to produce a pink colour which can be compared with either a natural or an artificial standard.

METHOD

An amount of well mixed fæces corresponding to about 1.5 g. is transferred by means of a glass rod to a 6 × 1 in. test tube. (This is easily done by weighing the glass rod before and after.) Water (9 ml.) is then added and the glass rod used to stir the mixture until it is well emulsified. 10 ml. of ferrous sulphate solution are added, well mixed and then 10 ml. of 2.5 N-sodium hydroxide. The mixture is allowed to stand for 2 hours with occasional stirring, and is then filtered.

Test. 2 ml. of filtrate (≡0.1 g. of fæces) are placed in a 100 ml. measuring cylinder and 2 ml. of Ehrlich's reagent added. After mixing and allowing to stand for 10 minutes, 6 ml. of sodium acetate solution are added (and an equal, or greater, volume of water, if the colour of the test is much greater than that of the standard).

Standard (≡0.00387 mg. urobilinogen per ml.). 1 ml. of phenolphthalein standard, in a 100 ml. volumetric flask, is treated with 5 ml. of sodium carbonate solution, diluted to the mark with water and mixed.

Blank. 2 ml. of the fæces filtrate, 2 ml. of 6N-hydrochloric acid and 6 ml. of sodium acetate are treated in the same way as the test.

With both photoelectric and Duboseq colorimeters a yellow green 625 filter is used.

The blank reading is subtracted from that of the test in the case of the photoelectric colorimeter.

CALCULATION

Photoelectric Colorimeter.

$$\text{Urobilinogen}^{*} \begin{cases} = \dfrac{\text{Reading of test} - \text{Reading of blank}}{\text{Reading of standard}} \times 0.00387 \times V \times \dfrac{100}{0.1} \\ = \dfrac{\text{Reading of test} - \text{Reading of blank}}{\text{Reading of standard}} \times V^{\dagger} \times 3.87 \end{cases}$$

* mg. per 100 g. fæces.

† Final volume of the coloured solution (ml.).

Duboscq Colorimeter. The calculation is the same as above, except that the ratio of readings is inverted to Reading of standard/Reading of test. The photometric method (pp. 197, 199) may be used with advantage.

SOLUTIONS

Ferrous Sulphate. 20 g. $FeSO_4.7H_2O$ dissolved in water and made to 100 ml.

2.5 N-*Sodium Hydroxide* (*approx.*). 10 g. of NaOH dissolved in water and made to 100 ml.

Ehrlich's Dimethylaminobenzaldehyde Reagent. 0.7 g. *p*-dimethylaminobenzaldehyde dissolved in a mixture of 150 ml. concentrated hydrochloric acid and 100 ml. water.

Sodium Acetate. Saturated solution of sodium acetate.

6 N-*Hydrochloric Acid.* 60 ml. of concentrated acid diluted to 100 ml. give approximately 6 N-HCl.

Standard Phenolphthalein Solution (Terwen, 1925) 50 mg. phenolphthalein dissolved in 100 ml. of alcohol and diluted 1 in 100 in alkaline solution as described above. This phenolphthalein standard has a similar colour to that given by 0.387 mg. urobilinogen in 100 ml. when treated by the above procedure (Watson, 1937), or to 0.00387 mg. in 1 ml.

Sodium Carbonate. 15 g. Na_2CO_3 dissolved in water and made to 100 ml.

Watson et al.'s (1944) *Standard.* An alternative standard solution, which more nearly matches the urobilinogen test, consists of 5 mg. of 'Pontacyl Carmine 2B' and 95 mg. of 'Pontacyl Violet 6R150 per cent' dissolved in 1 litre of 0.5 per cent acetic acid. When 10 ml. of this solution is diluted with 60 ml. of 0.5 per cent acetic acid a colour is obtained which is equivalent to that of 0.6 mg. of urobilinogen in 100 ml. when treated with Ehrlich's reagent.

Chapter VII

URINE

COLLECTION OF URINE

For most of the qualitative tests on urine, any freshly voided sample will suffice ; but it should be borne in mind that the first specimen passed in the morning is always the most suitable, since it has accumulated, and become concentrated, in the bladder during the night, and is likely to be the most concentrated of the 24 hours and the most uniform from day to day.

The quantities of urine passed at various times of the day are extremely variable : they may be large in volume and dilute in composition, as in the diuresis following meals or the drinking of fluids, or small and concentrated following a period when no water has been drunk. As a consequence, any quantitative estimation on a random sample is not of much use : the concentration of the determined constituent may be high or low, according as to whether the kidneys are excreting a concentrated or dilute urine. For quantitative work only 24-hour urine samples should be used ; and the amounts of the determined constituents should be expressed in terms of 24-hour output.

The collection of 24-hour specimens is not easy, since it requires the faithful co-operation of the patient, and the nursing staff if he be in hospital. The most satisfactory way of ensuring that all urine voided is preserved is to leave a stoppered Winchester bottle under the patient's bed, where he can urinate directly into it, or where the urine can be added immediately it has been passed in a receptacle in the bed. The bottle can then be collected and taken to the laboratory. A small amount of preservative—a few drops of chloroform or toluene, or a measured volume of a suitable acid—may with advantage be included in Winchesters for the collection of urine.

PROCEDURES FOR URINE

SUGAR

The most satisfactory solution for use in the clinical detection of sugar in urine is that devised by Benedict. It will keep indefinitely and is not affected by uric acid or creatinine which reduce some of the other reagents.

Benedict's Test. To 5 ml. of the reagent in a test tube are added 0.4 ml. (8 drops) of the urine to be tested. The mixture is boiled vigorously for 2 minutes in a bare flame, or 5 minutes in a boiling water-bath. In the absence of glucose the solution will remain perfectly clear or at most show only a faint turbidity or flocculent precipitate of phosphate. If clear print, in a good light, cannot be seen through the turbidity, the test may be considered positive. As the amount of sugar increases, the appearance of the precipitate varies from green to yellow and finally to orange with proportionate discharge of the original blue colour (see Table 8). (If much albumin is present in the urine it must be removed by precipitation with heat and acetic acid, followed by filtration.) The sugars, glucose, lactose, fructose and pentoses may be found in urine. Of these, glucose and lactose are the most common.

Distinction between Glucose and Lactose. Glucose and lactose both reduce Benedict's solutions. Lactose sometimes occurs in urine of pregnancy and lactation, and it is important to distinguish it from glucose. The following tests are used :—

(*a*) *Fermentation Test.* A large test tube is filled with urine, and a smaller tube, also filled with urine and containing a little yeast, is inverted in it. By closing the larger tube and inverting it carefully, the smaller tube is freed from air bubbles and sinks, in the inverted position, to the bottom of the large tube. In the presence of glucose fermentation will occur, and after a few hours the small tube will contain bubbles of carbon dioxide, the accumulation of which may bring it to the surface. If lactose alone is present no gas is produced.

(*b*) *Osazone Test.* To 5 ml. of the urine in a wide test tube are added 0.2 g. of phenylhydrazine hydrochloride, 0.4 g.

of sodium acetate crystals and 1 drop of glacial acetic acid. The mixture is heated for 45 minutes in a boiling water-bath, and is then filtered and allowed to cool slowly. Glucose and lactose each form characteristic yellow osazones, distinguishable by microscopic examination. Lactosazone is soluble in

TABLE 8

Colour of Precipitate and Supernatant when Benedict's Solution is Heated with Glucose

[0.4 ml. (≏8 drops) of glucose solution added to 5 ml. of Benedict's qualitative reagent. The tube is then placed in a boiling water bath for five minutes.]

Concentration of glucose solution (mg. per 100 ml.)	Observation
100	Slight turbidity ; faint coating of orange precipitate after settling ; deep blue supernatant.
200	Turbid with greenish tinge, blue colour of solution visible when held to light.
400	Greenish brown ; blue colour of solution visible when held to light.
600	Dark brown ; blue colour of solution visible when held to light.
800	'Nigger brown'; blue colour of solution *not* visible when held to light, but visible after red precipitate has settled.
(g. per 100 ml.)	
1.0	Chocolate brown ; brick red precipitate after settling ; blue supernatant.
1.5	Brown with trace of red ; brick-red precipitate ; slightly paler supernatant.
2.0	Reddish brown ; brick-red precipitate ; pale blue supernatant.
2.5	Brownish red ; brick-red precipitate ; very pale blue supernatant.
3.0	Bright-red ; brick-red precipitate ; orange-tinted supernatant.

hot water, and forms globules or clusters of fine crystals resembling fluffy yellow balls when the solution is cooled. Glucosazone is insoluble in both hot and cold water and gives lath-shaped crystals.

ESTIMATION OF GLUCOSE

In diabetes mellitus it is frequently useful to determine the amount of sugar excreted in the urine. The dosage of insulin may be gauged by the daily excretion of sugar, the patient

being under dietary control. The efficacy of the treatment may be most accurately determined by means of the estimation of the fasting blood sugar in conjunction with the above, but when facilities for the determination of blood sugar are not available it is possible to handle diabetes with reasonable success by the use of urine sugar estimations alone.

Meyer's Modification of Benedict's Method

The method affords a rapid and simple means for the estimation of sugar in urine.

5 ml. of Benedict's quantitative reagent together with 1 to 2 g. of anhydrous sodium carbonate are placed in a large test tube. About 5 ml. of distilled water are added to the mixture. The solution is brought to the boil over a small flame. The urine is added drop by drop from a 1 ml. graduated pipette. A few seconds' boiling is allowed between each addition of urine. The end point is recognized by the disappearance of the blue and green colour. 5 ml. of Benedict's solution are completely reduced by 10 mg. of glucose, 11.6 mg. of galactose, or 13.4 mg. of lactose. Fresh batches of Benedict's reagent should be tested with 1 g. per 100 ml. solutions of pure glucose and lactose (also with galactose if the reagent is to be used for the galactose excretion test).

SOLUTIONS

Composition of Benedict's Reagents

Qualitative Reagent. Sodium citrate (173 g. $Na_3C_6H_5O_7.11H_2O$) and anhydrous sodium carbonate (100 g. Na_2CO_3) are dissolved together in about 600 ml. of water. The solution, filtered if necessary, is poured into a 1 litre volumetric flask. A solution of copper sulphate (17.3 g.) in 100 ml. of water is added slowly with constant shaking. The volume is then made to 1 litre.

Quantitative Reagent. 100 g. of anhydrous sodium carbonate are dissolved in 600 ml. of hot water. Sodium citrate (200 g.) and potassium thiocyanate (125 g.) are added and dissolved with the aid of heat. The solution is filtered if necessary, cooled and poured into a 1 litre volumetric flask. A solution of copper sulphate (exactly 18.0 g. in 100 ml. of water) is then added gradually to the well shaken mixture, followed by 5 ml. of a 5 per cent solution of potassium ferrocyanide. The volume is made to 1 litre.

5 ml. of this solution should be equivalent to 10 mg. of glucose, but a freshly made solution should be standardized against a 1 per cent glucose solution.

'ACETONE'

The 'acetone' bodies in urine include acetoacetic and β-hydroxybutyric acids in addition to acetone.

Rothera's Test

To one-third test tube of solid ammonium sulphate are added not more than 5 ml. of urine and 8–10 drops of sodium nitroprusside solution (0.25 per cent in 1 per cent nitric acid), or a few granules of the solid. After the addition of 1–2 ml. of concentrated ammonia the tube is shaken well and allowed to stand. A permanganate colour indicates the presence of acetoacetic acid. If the quantity is small the colour may develop slowly.

This test detects 1 part of acetoacetic acid in 100,000 parts of urine.

Gerhardt's Test

To 5 ml. of urine is added, drop by drop, a solution of ferric chloride (3 per cent). White ferric phosphate is precipitated. More ferric chloride is added and the solution is filtered. A brown or purplish colour is obtained in the filtrate depending on the amount of acetoacetic acid present. The urine tested must be free from salicylic acid and salicylates and the compounds excreted after administration of aspirin, antipyrin, etc., all of which give a very similar reaction to acetoacetic acid, but never give the sodium nitroprusside reaction.

This test detects 1 part of acetoacetic acid in 7000 parts of urine. The colour, if due to acetoacetic, is discharged on heating; if due to salicylates, it persists.

Notes. When Rothera's test is found positive, Gerhardt's test should always be done in order that a rough estimate of the degree of ketosis may be made. When Rothera's test is negative no acetone bodies are present and other tests are unnecessary.

The nitroprusside reaction is a test for acetone, but acetoacetic acid decomposes so readily into acetone that it also

gives the test. The ferric chloride test for acetoacetic acid is not given by acetone.

SPECIFIC GRAVITY OF URINE

The urinometer is placed in the urine in the urinometer cylinder or a 50 ml. graduate. The urinometer should float freely in the urine and not touch the side of the vessel. In reading the urinometer the eyes should be on a level with the top of the meniscus of the urine both at the front and back of the cylinder. On the stem of the urinometer the number just visible above the surface of the urine is noted. This is the specific gravity to which 1000 is to be added.

ALBUMIN

Protein (in significant amount) in urine may be albumin and globulin (derived from the blood plasma) or 'Bence-Jones protein.'

Albumin and Globulin. These proteins are found in the urine (albumin greatly predominating in amount) both in 'normal' and pathological conditions. 'Normal' albuminuria occurs occasionally in youthful subjects and is generally increased in degree after exercise and decreased during rest. Thus, a morning specimen of urine may show a negative test for albumin, whereas one taken after exercise may contain considerable quantities of protein. This so-called 'orthostatic albuminuria' is not generally associated with renal or cardiac deficiency and frequently disappears in later life. The chief pathological conditions associated with albumin in the urine are the renal diseases such as the various forms of nephritis and nephrosis. The amount of protein voided is variable and the protein is mainly albumin.

'Bence-Jones Protein' is excreted in many cases of multiple myelomatosis and in other less well-defined conditions. Small amounts of protein excreted with blood, pus, or bile (without gross proteinuria) often give a positive test for 'protein.' In these cases, the amounts detected may be little more than the minimum detectable amount, namely about 10 mg. per 100 ml. of urine.

Heat and Acetic Acid Test

About 10 ml. of the urine (filtered if cloudy) are placed in a test tube. The tube is warmed in a small flame at a place about 3 inches above the bottom. A cloud will probably form at this place ; if this is so, 4–5 drops of dilute (5 per cent) acetic acid are added and the tube re-heated. If the cloud is due to phosphates it will now vanish ; if albumin is present the cloud remains and may increase.

If 'Bence-Jones Protein' is present, the cloud originally formed will vanish on cooling, reappear when heated to 60–70°C., and then disappear again at boiling temperature, where any albumin may be removed by filtration.

Sulphosalicylic Acid Test

1 ml. of clear urine (filtered if necessary) in a test tube is mixed with 3 ml. of sulphosalicylic acid solution (3 g. per 100 ml.). A precipitate indicates the presence of albumin.

NOTE. The above tests will detect albumin in urine in amounts as little as 10 mg. per 100 ml. Unlike the nitric acid tests they do not give 'false positive' results.

Quantitative Estimation of Albumin

The urine and sulphosalicylic acid are mixed in the proportions named above in a small tube of standard size. After 5 minutes, the turbidity is compared with that in the set of gelatin permanent standards (see page 97), and read as mg. of albumin per 100 ml. of urine. If the amount is greater than 100 mg. albumin per 100 ml. the urine is suitably diluted and a fresh estimation carried out.

BLOOD

Using boiled and cooled urine the benzidine or guaiacum test is carried out, as described on page 104. Urine containing blood frequently gives a positive test for albumin.

BILE PIGMENT

Urine containing bile is generally yellowish-green to brown in colour. When shaken it foams readily because of the

presence of the bile salts. The foam has a yellowish-green colour. This in itself is a rough test for bile, but the more accurate clinical tests depend upon the fact that bilirubin is oxidized by nitric acid to form a series of coloured compounds, biliverdin (green), bilicyanin (blue), choletelin (yellow), etc.

Nitric Acid Test

When a urine containing bile pigment is filtered the bilirubin is largely retained on the filter paper. 20 to 30 ml. of urine are filtered and *all* of it allowed to drain through the paper. The filter paper is unfolded and spread on top of the funnel. A yellow colour on the paper almost invariably indicates the presence of bilirubin. A drop of concentrated nitric acid is added to the flat paper. If bile pigments are present, the centre of the drop is coloured red and the fringes blue and green, indicating the presence of different oxidation products of the original yellowish-brown bilirubin.

Hunter's Test

2 ml. of 10 per cent barium chloride are added to 5 ml. of urine in a centrifuge tube. The precipitate of barium sulphate, on which the bile pigments are quantitatively taken up, is spun down and washed with a few ml. of water. 0.5 ml. of Van den Bergh diazo-reagent, 2 ml. of 95 per cent alcohol, and 0.5 ml. of sodium phosphate buffer ($Na_2HPO_4.12H_2O$—3.6 g. per 100 ml. of water) are added with thorough mixing after each addition. A red colour indicates bilirubin (Hunter, 1930) (cf. p. 37).

UROBILIN AND UROBILINOGEN

The amounts of urobilin which are usually present are too small to impart a colour to the urine of normal persons. Most of the pigment is present as the colourless urobilinogen which readily gives rise to urobilin on oxidation. Urines containing large amounts of urobilin are reddish in colour.

TESTS

Zinc Test. To 5 ml. of urine are added 2 drops of N/10 iodine solution followed by 5 ml. of a 10 per cent suspension

of zinc acetate in alcohol. The mixture is allowed to settle and in the clear supernatant a green fluorescence becomes apparent if there is urobilin or urobilinogen present. If a spectroscope is available, the fluid may be examined for the broad absorption band (due to urobilin) at the green-blue junction.

Copper Test. To 10 ml. of urine are added 0.5 ml. of glacial acetic acid, followed by 2 ml. of 5 per cent copper sulphate. 2 ml. of chloroform are added and the mixture thoroughly shaken and allowed to settle. If any urobilin is present it will appear in the chloroform layer, to which it imparts a pink colour. The spectrum may again be examined for absorption at the green-blue.

ESTIMATION

Ehrlich's test, using dimethylbenzaldehyde, may be applied to urine (see p. 109).

INDICAN

Only small amounts of indican, 5–20 mg. per day, appear in normal urine. Great increases are found in clinical conditions where there is stagnation and putrefaction of intestinal contents. Likewise decomposition of body proteins due to bacterial action, as in gangrene and putrid pus formation, results in large amounts of indican appearing in the urine.

TEST

To 5 ml. of urine in a test tube are added 5 ml. of concentrated hydrochloric acid and 2–3 drops of *dilute* sodium hypochlorite solution, or of a freshly made saturated solution of bleaching-powder (a little bleaching-powder shaken in 10 ml. of water in a test tube at room temperature). 2 to 3 ml. of chloroform are added and the test tube closed with the thumb and inverted several times. The amount of indican in a normal urine will give a light blue colour in the chloroform in this test: if indican is present in increased amount, the chloroform will be intensely coloured. The test is therefore 'controlled' with a normal urine, tested at the same time.

Care must be taken in the above test not to use a large excess of hypochlorite solution, or the blue-coloured chloroform-soluble product will be oxidized to a colourless substance.

MELANIN

The black melanin pigments do not occur in normal urine. Their occurrence is usually associated with melanotic tumours. The urine may be clear or somewhat darkened on passing, but on standing it rapidly deepens in colour and may become dark-brown or almost black.

Zeller's Test

When urine is shaken with an equal volume of bromine water a yellow precipitate forms which gradually darkens in colour and finally becomes black.

Ferric Chloride Test

On adding a few drops of ferric chloride to the urine in a test tube there is produced a grey colour followed by a black precipitate (consisting of ferric phosphate with adhering melanin) which re-dissolves with excess ferric chloride.

SULPHONAMIDES

With patients treated with sulphonamide drugs there is frequently precipitation of the sulphonamides as a crystalline precipitate in the urine. These crystals may be confused with those of uric acid and urates, but are easily distinguished by their solubility in acetone. Since much of the sulphonamides excreted in the urine is in conjugated form, it is necessary to hydrolyse them before applying the diazo colour reaction for their demonstration or estimation.

METHOD

A centrifuge tube is filled (10 or 15 ml.) with well shaken urine and is centrifuged until the precipitated material is thoroughly packed in the bottom of the tube. If the precipitate appears to occupy a volume of less than about 0.1 ml. the supernatant is discarded and a further tubeful of urine centrifuged in order to add its precipitate to the first.

The deposit is stirred and thoroughly mixed with 10 ml. of water, centrifuged, and the washing discarded. The tube is drained on filter paper, 5 ml. of acetone are added, and the precipitate stirred with a glass rod and the mixture carefully warmed under the hot water tap. The precipitate is allowed to settle while the tube is still warm, and the supernatant poured through a filter paper into a small (25 or 50 ml.) beaker. A further 5 ml. of acetone is added to the tube, warmed, mixed, and the further extract poured through the filter paper. The combined extracts are carefully evaporated to dryness on a hot water bath, and the residue then heated with 2.5 ml. of 0.2 N-hydrochloric acid, the beaker being covered with a watch glass, for 1 hour to hydrolyse the combined sulphonamides. The reaction and estimation for sulphonamide are then completed as described in the method for blood on p. 27.

NITROGENOUS CONSTITUENTS

UREA

Determinations of the different nitrogen constituents of urine are made according to procedures which are essentially the same as those used for blood.

Estimations of the amount of urea and of ammonia in the urine are valuable in a number of tests of renal function. The amount of urea in a single specimen may be from 0.02–4.00 g. per 100 ml., while the quantity of ammonia is usually small. The 24-hourly excretion is, approximately, 30 g. of urea and 0.8 g. of ammonia. In certain conditions the ammonia content of the urine may be greatly raised (e.g. in diabetic acidosis).

PRINCIPLES OF METHODS

(*a*) The urea is converted to ammonia with urease and the ammonia nitrogen estimated by Nesslerization.

(*b*) The urea is converted to nitrogen by sodium hypobromite and the nitrogen measured manometrically.

Method (*a*) is used for the accurate estimation of urea and ammonia, while method (*b*) is particularly well adapted for

use in renal function tests where a rapid and fairly accurate measure of the amount of urea+ammonia is required.

Urease-Nessler Method for Urea and Ammonia

PRINCIPLE

The urea is converted into ammonia with urease and the ammonia formed is estimated colorimetrically after Nesslerization. The preformed ammonia is determined in another sample and the urine urea content calculated by subtracting the ammonia content from the urea+ammonia content.

METHOD

A. Urea and Ammonia. *Test.* 1 ml. of urine is pipetted into a 50 ml. volumetric flask together with about 25 ml. of water. A 'knife-point' of Jack Bean meal is added and the flask stoppered, well-shaken and incubated at 37°C. for 20 minutes. 2 ml. of 10 per cent zinc sulphate and 2 ml. of N/2 sodium hydroxide are added and the volume of liquid is made to 50 ml. The well-mixed contents are allowed to stand 5 minutes and are then filtered. 1 ml. of the filtrate (≡0.02 ml. of urine) is pipetted into a 50 ml. volumetric flask.

Standards. Into two other 50 ml. volumetric flasks are pipetted 10 ml. and 20 ml. respectively of standard ammonium chloride solution (0.01 mg. N per ml.).

Water is added to each flask to about 40 ml., and after the addition of 5 ml. of Nessler's reagent more water is added to the 50 ml. mark and the solutions mixed. The test solution is then read in the colorimeter against the standard which is nearer in colour to it. The use of a violet (621) or blue (622) light filter facilitates the comparison.

CALCULATION

Photoelectric Colorimeter.

(1) Where the standard is 10 ml. of ammonium chloride,

$$\text{Urine urea+ammonia}^{*} \Big\} = \frac{\text{Reading of test}}{\text{Reading of standard}} \times 0.1 \times \frac{100}{0.02}$$

* mg. N per 100 ml.

(2) Where the standard is 20 ml. of ammonium chloride,

$$\text{Urine urea+ammonia*} \Big\} = \frac{\text{Reading of test}}{\text{Reading of standard}} \times 0.2 \times \frac{100}{0.02}$$

* mg. N per 100 ml.

To express the results as urea multiply by 2.14.

Duboscq Colorimeter. The calculation is the same as above, except that the ratio is inverted to Reading of test/Reading of standard (see p. 196).

B. Ammonia. 1 ml. of the urine is placed in a 50 ml. volumetric flask together with about 25 ml. of water; 2 ml. of sodium hydroxide and 2 ml. of zinc sulphate are added and, after diluting to the mark, the contents are filtered. 10 ml. of the filtrate (≡0.2 ml. of urine) are pipetted into another 50 ml. flask, water is added to about 40 ml. and, after the addition of 5 ml. of Nessler's reagent and dilution to the mark, the colour is read in a colorimeter in the same way and using the same standards as described for urine urea+ammonia.

CALCULATION

Photoelectric Colorimeter.

E.g., when the standard is 10 ml. of ammonium chloride,

$$\text{Urine ammonia*} \left\{ = \frac{\text{Reading of test}}{\text{Reading of standard}} \times 0.1 \times \frac{100}{0.2} \right.$$

* mg. N per 100 ml. (÷1.4=milli-equivalents per litre).

Notes. If urea only is required this is of course calculated by subtracting the ammonia value from urea+ammonia figure.

For the purpose of the urea clearance test the urea+ammonia figure is used since, as Van Slyke has pointed out, this result is a better index of the rate of urea excretion.

Hypobromite Method

The apparatus shown in Fig. 7 is used. The 50 ml. burette A is clamped vertically and its top end is closed with a doubly bored rubber bung. Through one of the holes in this

bung passes a short glass tube closed by a piece of pressure tubing and a clip B. Through the other hole passes a short tube connected with pressure tubing to the small bottle C, which is closed by a rubber bung. The other end of the burette is connected to the reservoir D, which is filled with water.

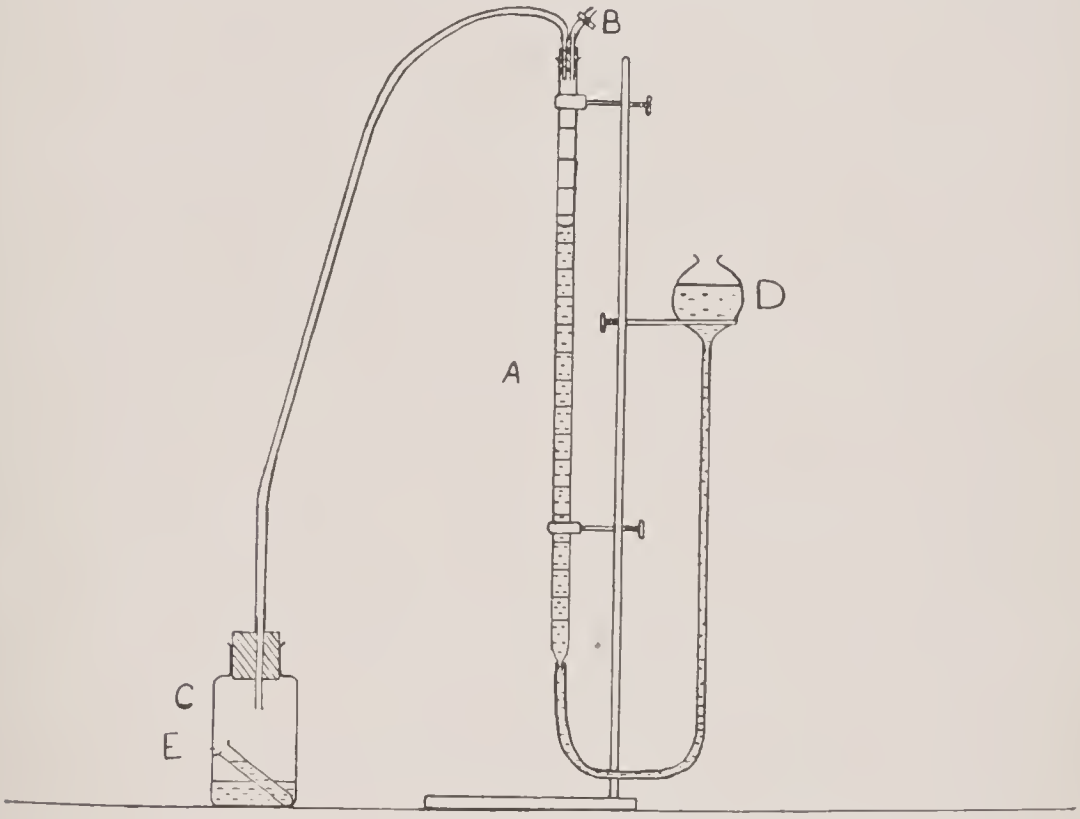


FIG. 7. Apparatus for determination of urea by hypobromite method.

METHOD

The estimation is carried out as follows : 10 ml. of sodium hypobromite solution are placed in the bottle C, and 2 ml. of urine in a small tube E, which rests in C. C is then tightly stoppered, and placed in a large beaker of water for 5 minutes. The clip B is opened and the water level is adjusted to zero by moving D. B is then closed, and the contents of E mixed with the hypobromite by tipping. C is now well shaken until reaction ceases, when C is placed in the beaker for a further 5 minutes. The level of water in the burette is now read with the level of water in D at the same height. This reading is the volume of nitrogen evolved, under the conditions, from 2 ml. of urine.

CALCULATION

The theoretical reaction is as follows :—

$$CH_4N_2O + 3NaOBr + 2NaOH \longrightarrow N_2 + 3NaBr +$$
urea, 60 g. (22.4 l.) $Na_2CO_3 + 3H_2O$.

Hence, 60 g. urea ≡ 22.4 l. of nitrogen at N.T.P.

∴ 1 g. „ ≡ 373 ml. „ „ „ „

In fact, 357 ml. of nitrogen are yielded on the average by 1 g. of urea, and it is not necessary to correct for temperature and pressure.

Hence, 357 ml. N_2 = 1 g. Urea

$$\therefore \quad \text{Urea in urine}^* = \frac{\text{ml. } N_2}{357} \times \frac{100}{2}$$

$$= \frac{\text{ml. } N_2}{7.14} = \text{ml. } N_2 \times 0.14$$

* g. per 100 ml.

Reagent. Sodium hypobromite (prepared fresh, daily). 2.5 ml. of bromine are carefully added to 25 ml. of sodium hydroxide (40 g. per 100 ml.).

AMINO ACIDS

There are normally present small amounts of amino acids in the urine. Their nitrogen accounts for about 1 per cent of the total urinary nitrogen, and corresponds to an excretion of 100–150 mg. of amino nitrogen per day. Increased amounts are found in typhoid and in other conditions associated with wastage of tissues ; also in cystinuria and in Fanconi syndrome (Dent, 1947), and in severe liver disease.

PRINCIPLE

Solutions of amino acids are nearly neutral in reaction. The addition of formaldehyde, however, destroys the amino group and the acids so formed can be titrated with sodium hydroxide. Ammonia also enters into this reaction, and results so obtained include the ammonia as well as amino

aeids. The ammonia may be removed by means of phosphotungstic acid or it may be separately determined (see p. 124). For most purposes, however, the eombined results, of amino aeids, *plus* ammonia, are suffieiently useful. The end-point of the titration is obseured by the presence of mueh phosphate; but this is easily removed through the insolubility of its barium salt.

METHOD

25 ml. of the urine are pipetted into a 50 ml. volumetric flask and 0.5 ml. of phenolphthalein indicator (see p. 191) and 2 g. of solid barium chloride are added. The mixture is shaken until the barium chloride is in solution, and N-sodium hydroxide is then added until pink to phenolphthalein. The flask is filled to the mark with freshly distilled water, well mixed and the mixture filtered after 15 minutes. 40 ml. of the elear filtrate are plaeed in another 50 ml. volumetric flask, the pink eolour diseharged by the addition of a little N-hydrochlorie acid and the solution then diluted to the mark.

25 ml. of this solution (≡10 ml. of urine) are treated with 0.1 N-sodium hydroxide until the pink eolour of phenolphthalein appears. 10 ml. of neutralized formaldehyde are now added, the solution mixed (the pink eolour will be seen to disappear), and the solution titrated with 0.1 N-sodium hydroxide until alkaline to phenolphthalein.

CALCULATION

$$\text{Amino nitrogen}^* = \text{ml. } 0.1 \text{ N-NaOH} \times 0.0014 \times \frac{100}{10}$$

* in g. amino N per 100 ml. of urine. This result includes ammonia nitrogen, whieh, if desired, can be determined separately and the result subtracted from the above in order to obtain the true amino acid figure. The result should be calculated and expressed in g. per 24 hours output of urine.

SOLUTIONS

0.1 N-*Sodium Hydroxide.* See p. 187.

Phenolphthalein Indicator. See p. 191.

Neutral Formaldehyde. To 25 ml. of commercial formaldehyde solution (formalin), which is a 40 per cent solution of formaldehyde, are added 0.5 ml. phenolphthalein indicator and 0.1-N-sodium hydroxide until the solution is distinctly pink.

TOTAL NITROGEN OF URINE

The nitrogen-containing compounds of urine comprise ammonium salts, urea, uric acid, creatinine and small amounts of other substances, some of them still unidentified. The total nitrogen excreted by a normal adult individual is about 14 g. per day. This amount may be depressed in conditions involving kidney damage.

PRINCIPLE

The Kjeldahl Method

The organic substances of the urine are destroyed by digestion with concentrated sulphuric acid and all the nitrogen present is converted into ammonium sulphate. By the addition of excess of alkali, ammonia is liberated and is quantitatively distilled off into a measured amount of standard acid. The amount of acid neutralized by the ammonia gives the measure of the amount of nitrogen present in the sample of urine used.

METHOD

1 ml. of urine is accurately measured into one of the small digestion flasks of the micro-Kjeldahl outfit. 2 ml. of concentrated sulphuric acid are added and 0.5 g. of potassium sulphate, a 'knife point' of copper sulphate and selenium dioxide. The mixture is heated on the special digestion apparatus (Fig. 2, p. 43). It gradually turns black as the organic material is charred, then becomes light brown, and finally a clear blue. The flask is heated for 2 hours, cooled to room temperature and the contents diluted with 10–20 ml. of distilled water. The solution is quantitatively transferred to the distilling apparatus (Fig. 3, p. 47) by pouring it through the small funnel attached to *g* and following by successive washings of the flask with small amounts of distilled water. 10 ml. of 0.1 N-sulphuric acid are now added to a 100 ml. conical flask. 2 drops of methyl red indicator (or of the Tashiro's indicator described on p. 47) are added and the flask placed on the stand beneath the condenser tube of the

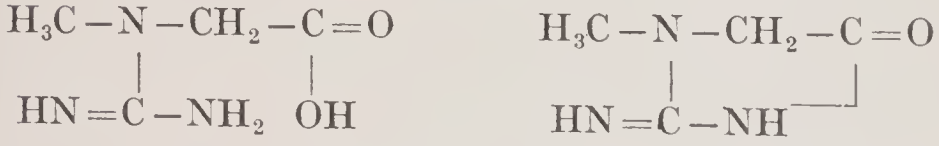
distillation apparatus. The height of the flask is adjusted so that the bottom of the distillation tube dips below the surface of the liquid (*p*). 15 ml. of concentrated alkali (40 per cent sodium hydroxide) are now added to the distillation apparatus through the small funnel. The ammonia which is liberated is distilled over (through *c* and *f*) into the standard acid in the conical flask by bubbling steam (from *A*, via *l*, *k*, *s*, *h*, and *e*) through the mixture (*a*). A Bunsen burner is placed under the large round bottomed boiling flask *A* which should be about half full of water. Steam is bubbled through the mixture and the distillation continued until the contents of the conical flask are about twice what they originally were. The flask is now lowered until the end of the condenser tube (*p*) no longer dips into the liquid. Heating is continued and steam passed for about a minute. The condenser tube is washed down with distilled water into the conical flask, which is now removed and its contents titrated with 0.1 N-sodium hydroxide. The titration figure so obtained is subtracted from the 10 ml. of 0.1 N-sulphuric acid to give the ml. of standard acid which were neutralized by the ammonia.

CALCULATION

ml. 0.1 N-H_2SO_4 neutralized by ammonia $\times 1.4$
$=$mg. nitrogen in the 1 ml. of urine.

It is usual to express the nitrogen content of urine in terms of the output per 24 hours.

CREATINE AND CREATININE



Creatine is methyl-guanidine-acetic acid.

Creatinine is its anhydride.

The excretion of *creatinine* in the normal individual varies according to the muscular weight. The amount eliminated each 24 hours ranges between 0.4 and 1.8 g. in the adult. On a creatine-free diet the output is remarkably constant and very seldom varies except in uncommon clinical conditions.

The excretion of *creatine* in normal adult females is intermittent; and in adult males creatine is very seldom present and only in small quantities. It is increased in the urine in any condition which raises the B.M.R. in the myopathies and in several endocrine disturbances.

METHOD FOR CREATININE

The Folin method is based upon the production of an orange-red colour by the interaction of creatinine with alkaline sodium picrate. The colours produced are compared in a colorimeter, and the creatinine content of the urine estimated by comparison with a creatinine solution of known strength.

Test. A volume (usually 1–3 ml.) of urine containing 0.7 to 1.5 mg. creatinine, is measured with a pipette into a 100 ml. volumetric flask.

Standard. Into a similar flask is pipetted 1 ml. (≡1 mg. of creatinine) of 'stock' standard creatinine solution.

To each flask are added 1 ml. of N-sodium hydroxide and 2 ml. of saturated picric acid. After 10 minutes each flask is filled to the mark with water and the contents mixed. [For photoelectric comparison, a 'blank' (water and reagents) is used for the zero setting.] A mercury green light filter (807) is used for photoelectric colorimeters, a blue green (623) for the Duboscq.

CALCULATION

Photoelectric Colorimeter.

$$\text{Creatinine}^* \begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 1.0 \times \dfrac{100}{v} \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times \dfrac{100}{v} \end{cases}$$

where v is the volume of urine used.

* mg. per 100 ml. of urine.

Duboscq Colorimeter. The calculation is the same as above except that the ratio of readings is inverted to Reading of standard/Reading of test.

METHOD FOR CREATINE

Boiling Method. Creatine is converted to creatinine by boiling in picric acid solution. 20 ml. of saturated picric acid solution are pipetted into a flask together with a measured amount of urine (usually equal to the volume used for the creatinine determination), and about 130 ml. water. The contents are brought to the boil rapidly, and then allowed to boil gently for 1 hour. The flame is raised and the mixture boiled down rapidly to bring the volume of the solution to about 25–30 ml. The flask is cooled, and 4 ml. N-sodium hydroxide are added. After 10 minutes the contents of the flask are transferred to a 100 ml. volumetric flask and water is added to the mark. The contents are compared in the colorimeter with the standard coloured solution used for creatinine.

Autoclave Method. Creatine may be turned into creatinine by heating its solution under pressure with a strong mineral acid. A determination with the alkaline picrate method then gives the sum of creatinine and creatine. 1 ml. of urine and 1 ml. of N-hydrochloric acid are autoclaved for 20 to 30 minutes at 120°C. and 14 lb. pressure. 2 ml. of picric acid solution and 2 ml. of N-sodium hydroxide are added; and, after 10 minutes, water to 100 ml. The colour is measured and compared with that of the creatinine standard.

CALCULATION

As for creatinine. The result is total creatinine, i.e. preformed creatinine plus creatine, expressed as creatinine. Thus, the creatine content of urine (expressed as creatinine) = total creatinine − preformed creatinine.

SOLUTIONS

N-Sodium Hydroxide.

Saturated Solution of Picric Acid. About 1.5 per cent (see p. 17).

Creatinine 'Stock' Standard (containing 1 mg. of creatinine per ml.). 1.602 g. of pure creatinine zinc chloride are dissolved in N/10 hydrochloric acid solution, and the volume made up with the N/10 acid to 1 litre. (This is the same *'Stock' Standard* as is used in the blood method, p. 17.)

URIC ACID

Urine contains about 0.4 g. of uric acid per 24-hourly specimen. This amount may be increased in cases of leukæmia, and decreased in gout.

PRINCIPLE

Urine is diluted and treated with Benedict's arsenophosphotungstic acid reagent and then with a sodium cyanide-urea solution. The blue colour produced by reduction, at the alkaline reaction, of the arsenophosphotungstic acid, is compared with that obtained under similar conditions from a standard uric acid solution.

METHOD

The sample of urine should be fresh in order to avoid loss of uric acid through precipitation of urates which takes place on standing. Samples of urine which have deposits should be warmed until all precipitated matter has dissolved.

Test. 1 ml. of urine is diluted to 25 ml. in a volumetric flask. 5 and 10 ml. of this solution (≡0.2 and 0.4 ml. of urine) are placed in 50 ml. volumetric flasks.

Standards. 5 ml. and 10 ml. of the uric acid urine standard (≡0.1 and 0.2 mg. uric acid) are placed in similar flasks.

To each mixture are added 5 ml. of urea-cyanide solution, and 1 ml. of Benedict's uric acid reagent. After 10 minutes, the solutions are made to the mark and mixed. The standard and nearest test are compared in a colorimeter with a red (608) light filter.

CALCULATION

Photoelectric Colorimeter (0.2 ml. test and 0.1 mg. standard).

$$\text{Uric acid}^* \begin{cases} = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 0.1 \times \dfrac{100}{0.2} \\ = \dfrac{\text{Reading of test}}{\text{Reading of standard}} \times 50 \end{cases}$$

* mg. per 100 ml. of urine.

Duboscq Colorimeter (0.4 ml. test and 0.2 mg. standard).

$$\text{Uric acid}^* \begin{cases} = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 0.2 \times \dfrac{100}{0.4} \\ = \dfrac{\text{Reading of standard}}{\text{Reading of test}} \times 50 \end{cases}$$

* mg. per 100 ml. of urine.

NOTE. Depending on the concentration of uric acid, it may be advisable to use more or less of the diluted urine than is specified above.

SOLUTIONS

Benedict's Arsenophosphotungstic Acid Reagent. 100 g. of sodium tungstate are dissolved in about 600 ml. of water in a round-bottomed litre flask. To the solution are added 50 g. of pure arsenic pentoxide followed by 25 ml. of syrupy phosphoric acid (85 per cent) and 20 ml. of concentrated hydrochloric acid. This mixture is boiled for 20 minutes, cooled, washed into a 1 litre volumetric flask and diluted to the mark.

Stock Uric Acid Standard (Folin) (≡1.0 *mg. per ml.*). (See p. 14.)

Uric Acid Urine Standard (0.02 *mg. per ml.*). 20 ml. of the above standard are diluted with 1 ml. of 40 per cent formalin and water to 1 litre.

Urea-cyanide Solution. 5 g. of sodium cyanide and 20 g. of urea are dissolved in water, and the volume made to 100 ml.

CHLORIDES

The usual excretion of chloride amounts to about 10 or 12 g. (expressed as sodium chloride) per 24 hours for normal individuals. The amount of this excretion may be altered during certain clinical conditions. It is most markedly depressed in conditions of œdema which are characterized by salt and water retention.

PRINCIPLE

The Volhard procedure for the determination of chloride in solution consists in the precipitation of the chloride with a measured excess of standard silver nitrate in the presence of nitric acid. The excess of silver nitrate over that necessary for complete precipitation is measured by titration with

standard ammonium thiocyanate. A ferric salt is added as indicator, the first production of a red colour due to the formation of ferric thiocyanate indicating the point at which an excess of ammonium thiocyanate has been added.

$$NaCl + AgNO_3 = NaNO_3 + AgCl$$
$$AgNO_3 + NH_4CNS = AgCNS + NH_4NO_3$$

The difference between the volume of the standard silver nitrate used and that of the standard ammonium thiocyanate represents the amount of silver precipitated by the chloride.

METHOD

10 ml. of urine are added to about 25 ml. of water in a 100 ml. volumetric flask. 20 ml. of the standard silver nitrate are added and after a few minutes the volume made to the 100 ml. mark. The mixture is filtered, and 50 ml. of the clear liquid are transferred to a 250 ml. conical flask for titration. 5 ml. of ferric alum are added to serve as indicator. Standard ammonium thiocyanate is run in until the production of the first permanent faint reddish-pink colour.

CALCULATION

The figure representing the thiocyanate titration is subtracted from 10 (ml. of silver nitrate) to give the figure representing the silver nitrate precipitated by chloride.

1 ml. standard N/5.85 silver nitrate precipitates 0.01 g. sodium chloride.

$\therefore$ (10 − ml. thiocyanate) × 0.01 = g. sodium chloride in 5 ml. of urine (i.e. in half the volume taken).

It is usual to express the chloride content of urine in terms of the output of sodium chloride per 24 hours, either as grams or as milli-equivalents (g. × 1000/58.5).

SOLUTIONS

Standard N/5.85 *Silver Nitrate.* 29.060 g. silver nitrate crystals are dissolved in about 400 ml. of water in a litre flask. 85 ml. of concentrated nitric acid (sp. gr. 1.43) are added, mixed, and made

to 1 litre. This standard silver nitrate solution is of such a concentration, i.e. N/5.85, that 1 ml. is the equivalent of 0.01 g. sodium chloride (molecular weight of NaCl=58.5). It may be diluted 1 in 10 for C.S.F. chloride.

Ferric Alum. 4 g. ferric ammonium sulphate (alum) are dissolved in 100 ml. of water.

Standard Ammonium Thiocyanate. This solution is prepared in such a manner that 1 ml. of it is equivalent to 1 ml. of the standard silver nitrate solution used. 13 g. of ammonium thiocyanate (NH_4SCN) are dissolved in 1 litre of water. This gives an approximately N/5.85 solution. Some of it is titrated from a burette into 10 ml. of the standard silver nitrate solution, with the addition of ferric alum, in order to determine its exact concentration. The remaining solution is then suitably diluted to make it exactly N/5.85.

SODIUM

The method of estimating sodium in blood plasma is applied to a sample of the urine from which the phosphate has been precipitated with calcium hydroxide.

To about 10 ml. of urine in a small flask, approximately 0.25 g. of dry powdered $Ca(OH)_2$ are added. The mixture is well shaken and is filtered clear. The filtrate should be protected from the air in a stoppered test tube, to prevent precipitation of $CaCO_3$ from the action of CO_2 on the excess $Ca(OH)_2$. 0.5 ml. of the filtrate is treated with 1.5 ml. of 7 per cent trichloroacetic acid, filtered if necessary, and 0.1 and 0.2 ml. of the filtrate are used for the zinc uranyl acetate method for sodium in the same manner as blood plasma (p. 51).

POTASSIUM

The cobaltinitrite method for blood serum is applied to a sample of the urine from which the ammonia, which forms an insoluble cobaltinitrite like potassium, has been eliminated by ashing and re-solution in dilute acid.

2 ml. of urine are evaporated to dryness in a good quality porcelain, vitreosil or platinum crucible. The residue is ashed at a dull red temperature (about 400°C.) for some hours. Overnight is convenient if an electric muffle is available ; otherwise the crucible may be placed inside two porcelain

evaporating dishes, one inverted over the other, and heated with a Bunsen burner until all carbonaceous matter has disappeared.

The ash is dissolved in a small amount of 0.1 N-hydrochloric acid, and the solution transferred quantitatively with washings of water to a 10 ml. standard flask, made to volume and mixed. (If the potassium content is likely to be high, the solution of the ash is made to 15 or 20 ml.)

0.2 ml. of this solution is used for the potassium determination described for blood serum (p. 86). The result so obtained is multiplied by 5 to allow for the dilution of 2 ml. of urine to 10 ml.

CALCIUM

The method described for calcium in blood serum may be used.

PHOSPHATE

2 ml. (plus 3 ml. water) and 5 ml. of 1 in 100 diluted urine are treated with perchloric acid, ammonium molybdate and aminonaphtholsulphonic acid (p. 65).

DIASTASE

The estimation of urinary diastase is of some importance in diseases of the pancreas, in which values higher than normal are found. The 'units' used are those of Wohlgemuth. They are given by the number of ml. of 0.1 per cent starch solution digested by 1 ml. of urine in 30 minutes. Expressed in these terms, the normal values are 6–30 units per ml. In pancreatic abnormalities the value may rise to over 100 units.

PRINCIPLE

Different dilutions of the urine, buffered to pH 6.1 with phosphate solution, are mixed with a 0.2 per cent solution of starch. After incubation at 38°C., iodine is added to each sample, and the last tube in which no colour is produced is taken as giving the dilution at which the starch is just completely digested.

METHOD

1 ml. of the urine is added to 4 ml. of the buffer solution (pH 6.1), making a 1 in 5 dilution of the urine. Seven small test tubes are then placed in a rack and 4 ml. of the buffered urine (of dilution 1 in 5) are placed in tube (1). 2 ml. of buffer are added to each of tubes (2) to (7). 2 ml. from tube (1) are mixed with the contents of tube (2). 2 ml. of this mixture from tube (2) are transferred to tube (3), and so forth until tube (7) is reached, from the final contents of which 2 ml. are discarded. The dilutions are then 1 in 5 in tube (1), 1 in 10 in tube (2), 1 in 20 in tube (3), and so on, to 1 in 320 in tube (7).

1 ml. of 0.2 per cent starch solution is then added to each tube and mixed with the other liquids. The tubes are incubated, by immersion in a water-bath, at 38°C. for 30 minutes. At the end of this time 3 drops of N/50 iodine are added to each tube.

CALCULATION

Let the dilution of the urine in the last tube which shows no blue or mauve colour be 1 in x.

This tube contains :—

2 ml. of a 1 in x dilution of urine, i.e., $\frac{2}{x}$ ml. of urine.

Therefore $\frac{2}{x}$ ml. of urine contains just sufficient diastase to digest 1 ml. of 0.2 per cent starch solution in 30 minutes ; that is,

$\frac{2}{x}$ ml. of urine digests 2 ml. of 0.1 per cent starch solution ; hence,

1 ml. of urine digests $\frac{2}{2/x} = x$ ml. of 0.1 per cent starch solution.

Therefore, the number of Wohlgemuth units per ml. is equal to x, the dilution factor of the tube in which the digestion of the starch is just complete.

Urinary diastase is usually reported in terms of the output of the enzyme for 24 hours.

SOLUTIONS

Phosphate buffer (*p*H 6.1). This is made by mixing two solutions A and B.

Solution A. 11.876 g. of disodium phosphate ($Na_2HPO_4.2H_2O$) are dissolved in freshly boiled distilled water, and the volume made to 1 litre.

Solution B. 9.078 g. of potassium dihydrogen phosphate are dissolved as above, and the volume made to 1 litre.

These two solutions are kept in bottles lined with paraffin-wax. The phosphate buffer pH 6.1, used in the method, is made by mixing 15 ml. of solution A with 85 ml. of solution B.

Starch Solution (0.2 *per cent*). 0.2 g. of starch is shaken in a test-tube with a little cold water. The paste so formed is washed into about 80 ml. of boiling water in a beaker.

When the solution is cool, the mixture is poured and washed into a measuring flask, and the volume made to 100 ml.

0.1 N-*Iodine.* This contains 12.7 g. of iodine crystals dissolved in a solution of 20 g. of potassium iodide in a little water. The volume is then made to 1 litre (see p. 191).

N/50 *Iodine.* This is made by appropriate dilution of the 0.1 N-iodine with water.

ASCORBIC ACID

The 24-hourly excretion of urinary ascorbic acid in normal persons (whose intake is 20–80 mg. per day) is in the neighbourhood of 50 per cent of the intake. In cases of vitamin C deficiency and in certain infections there may be little or no ascorbic acid in the urine (Harris and Ray, 1935).

COLLECTION OF URINE

Urine is collected during 24 hours. Day and night specimens are put as soon as voided into a bottle containing exactly 100 ml. of glacial acetic acid and well mixed with the acid. All urine is analysed as soon as possible.

PRINCIPLE

Ascorbic acid rapidly reduces, in acid solution, the dye 2 : 6-dichlorophenol-indophenol to a colourless substance.

Urines containing ascorbic acid also effect this reduction and the amount of dye reduced by the urine is a fairly accurate measure of the quantity of the acid present.

METHOD

An appropriate amount of the dye (usually 0.5 ml. of the solution of 2 : 6-dichlorophenol-indophenol) is measured into a small test tube. A drop of 50 per cent acetic acid is added and the urine rapidly run in from a 2 ml. burette until the red colour of the dye disappears.

CALCULATION

0.5 ml. of 2 : 6-dichlorophenol-indophenol solution contains 0.04 mg. of dye. This amount is reduced by approximately 0.02 mg. ascorbic acid. For accurate work, the dye solution should be checked against the pure vitamin and should be freshly prepared daily.

The amount of ascorbic acid equivalent to the dye taken is contained in the quantity of urine used to decolorize the dye, whence the amount present in the total sample or in 100 ml. of urine is calculated. Where acetic acid has been added as preservative the volume added must be allowed for in the calculation. Results are expressed in mg. ascorbic acid per 100 ml. of urine or as mg. excreted in 24 hours, according to the information desired and the type of test being conducted.

Example. A sample of urine had a total volume of 720 ml. including 100 ml. of glacial acetic acid.

0.5 ml. of dye solution required 0.82 ml. of the acidified urine

$\therefore$ 0.82 ml. of the acidified urine contained 0.02 mg. of ascorbic acid

Hence 720 ml. of the acidified urine contained $\frac{0.02}{0.82} \times 720$

$= 17.6$ mg.

Since 17.6 mg. of ascorbic acid were contained in 720 ml. of acidified urine, this amount would also be contained in 620 ml. (720 − 100) of unacidified urine.

$\therefore$ 100 ml. of unacidified urine contained $\left\} \frac{17.6 \times 100}{620} = 2.8 \right.$ mg. ascorbic acid.

SOLUTION

20 mg. (accurately weighed) of pure dichlorophenol-indophenol dye are dissolved in 250 ml. of water. Alternatively one tablet (2 mg. of the dye≡1 mg. ascorbic acid ; Messrs. Hoffman—La Roche or British Drug Houses) is placed in a 25 ml. volumetric flask : about 10 ml. of warm water are added, and after the tablet has dissolved the contents of the flask are cooled and diluted to the mark. The resulting solution contains 0.04 mg. of dichlorophenol-indophenol per 0.5 ml. (≡0.02 mg. ascorbic acid).

ANEURIN, VITAMIN B_1

PRINCIPLE

The method of estimating aneurin is that described by Wang and Harris (1939, 1943), and by Simmons and Wootton (1944). It depends on the transformation of aneurin to thiochrome and comparison by fluorescence against a similarly treated known standard of aneurin. Normal values of urinary aneurin are variously given as 33–130 I.U. (=99–390 μg. per day), 50–80 I.U. (=150–240 μg. per day), 30–160 I.U. (=90–480 μg. per day). (I.U.=International Unit=3 μg.)

METHOD

The specimen of urine is adjusted to approximately pH 3 as soon as possible. To 8 ml. of urine an equal amount of *iso*-butanol (saturated with water) is added in a 25 ml. glass-stoppered cylinder and shaken for 2 minutes. This extracted urine is used for the estimation. The butanol (upper layer) is discarded.

It is important that strict attention should be paid to the following details if reliable results are to be obtained. Four cylinders are marked : A and B (blanks), T (test) and S (standard), and the indicated amounts of urine, etc., placed in them.

To cylinder A is added 2 per cent potassium ferricyanide drop by drop from a burette until the colour remains slightly

	A	B	T	S
Extracted urine	2 ml.	2 ml.	2 ml.	1 ml. of dilute standard
Methanol	2 ml.	2 ml.	2 ml.	0.5 ml. water, 2 ml. of methanol.
20% sodium hydroxide	1 ml.	1 ml.	—	—

more yellow than cylinder B for 30 seconds. The volume added is noted, and this tube is discarded. The remaining cylinders are treated as follows :—

	B	T	S
2% potassium ferricyanide	—	Same amount as used in tube A	1 drop
20% sodium hydroxide	—	1 ml. (mix)	1 ml. (mix)
Hydrogen peroxide 5% (20 vol.)	5 drops	5 drops	5 drops
Iso-butanol	10 ml.	10 ml.	10 ml.

All three cylinders are shaken vigorously for 2 minutes and the mixtures allowed to separate ; the aqueous lower layers are removed and discarded. 4 ml. distilled water are added to each cylinder and shaken for 2 minutes, allowed to separate, the water removed and 2 ml. of ethanol added to clarify, The volumes of B, T and Standard are made up to 15 ml. with redistilled *iso*-butanol, and 10 ml. of B and T are used for matching fluorescence.

Fluorimetry. Two tubes are labelled B and T. 10 ml. of the *iso*-butanol extract B are measured into tube B, and a similar quantity of T into tube T. The comparison is made in a completely dark room using an ultra-violet lamp, which is enclosed in a case fitted with a Woods glass filter. The tubes are held in front of the lamp and the fluorescence viewed down the long axis of the tubes. The bottoms of the tubes rest on a blackened platform fitted to the case of the lamp. Precautions are taken to interchange the position of the tubes and to inspect the fluorescence quickly to avoid errors due to fatigue. Standard thiochrome (S) is added to tube B drop by drop with mixing, and at the same time

an equal volume of redistilled *iso*-butanol is added to tube T, until the fluorescence in each tube is adjudged equal. Possible sources of error to be eliminated include irregularities in the size and uniformity of the tubes and the presence of fluorescing substances in the glass or the reagents. It should be noted that aspirin, quinine and other drugs may cause abnormal fluorescence. The 'Hysil' test tubes manufactured by Messrs. Chance Bros. are made of non-fluorescent glass, and are suitable for the comparison.

CALCULATION

$$\left.\begin{matrix}\mu\text{g. Aneurin in} \\ 2\text{ ml. of urine}\end{matrix}\right\} = \frac{\text{Total solution}}{\text{Amount used in test}} \times \frac{x\text{ ml. of standard used}}{\text{Volume of standard}}$$

$$= \frac{15}{10} \times \frac{x}{15} = \frac{x}{10}$$

It is usual to conduct the analysis on a sample of a 24-hour specimen of urine, and to express the result as the total output in micrograms (μg.) of aneurin for the 24-hour period.

SOLUTIONS

Preparation of Standard. 'Benerva' ampoules are used (Roche Products, Ltd.). 10 mg. are dissolved in 100 ml. of N/10 hydrochloric acid in 25 per cent alcohol. 10 ml. of this solution are diluted with N/10 hydrochloric acid (aqueous) to 1 litre. This dilute solution should be prepared fresh daily for use ($\equiv$1 mg. per 1000 ml. or 1 μg. per ml.).

17-KETOSTEROIDS

The estimation of the daily excretion of 17-ketosteroids is useful in the study of conditions associated with several endocrine abnormalities. For normal men the 24-hour output is between 11 and 23 mg., and for women 4–24 mg. (Barnett, Henly, Morris and Warren, 1946). Some authors have recorded a lower range, particularly for males (4–14 mg.; Callow, Callow, Emmens and Stroud, 1939). Barnett *et al.* give a summary of the normal values given in the literature. Children excrete only small amounts: 1–2 mg. (4–7 years), about 4 mg. (7–12 years) and 8–10 mg. (12–15 years). These and subsequent values are in mg. per 24 hours.

Low values have been recorded by various workers for Simmond's disease (0), anorexia nervosa (low, but not zero), Addison's disease in women, cretinism and pituitary infantilism. Abnormally high excretions have been found to occur in cases of adrenal cortical tumours (very high, >100), adrenal hyperplasia (40–100 mg.), testicular tumour, Cushing's syndrome (slightly raised, up to 40 mg.). A discussion of abnormal values is given by Pincus and Thimann (1948), and by Scowen and Warren (1946).

PRINCIPLE

The ketosteroids are present in urine largely as water-soluble conjugates ; these conjugates are hydrolysed by acid to the free steroids, which are then extracted with carbon tetrachloride. 17-ketosteroids (androgens) produce a pink colour in alcoholic potassium hydroxide solution with *m*-dinitrobenzene. This is compared with the colour produced with a suitable androsterone standard.

METHOD

The following procedure is taken from the method of Robinson and Warren (1947).

Collection of Urine. It is essential that a complete 24-hour sample of urine be obtained (see p. 112). No phenolic preservatives should be used. It is advisable to add nothing other than 10 ml. of concentrated hydrochloric acid (Analar) and a little copper sulphate (1 g.) to the Winchester bottle into which the urine is to be put.

Extraction of Urine. Robbie and Gibson's (1943) method is used. 100 ml. of the urine in a 250 ml. flask are treated with 10 ml. of concentrated hydrochloric acid and boiled for 10 minutes under a reflux condenser. The flask is cooled and 30 ml. of carbon tetrachloride are added through the condenser. The mixture is again boiled for 10 minutes. It is cooled, transferred to a separating funnel and the carbon tetrachloride run off. 20 ml. of additional carbon tetrachloride are added and the mixture well swirled (not shaken). The carbon tetrachloride layer is allowed to separate, and is drained. 10 ml. of fresh carbon tetrachloride are added,

the mixture well swirled again, separated and the carbon tetrachloride layer added to the first and second extracts.

The 60 ml. of combined carbon tetrachloride extracts are washed in a clean separating funnel with 20 ml. of water, then with 20 ml. of 2 N-sodium hydroxide to remove acids and phenols, and finally with two changes of 20 ml. of water.

The washed carbon tetrachloride extract is carefully evaporated to dryness in a 100 ml. flask on a water bath. It is convenient to use a water vacuum pump to remove the last traces of carbon tetrachloride.

The residue is dissolved in 4 ml. of absolute alcohol added to the warm flask, which is stoppered and shaken by swirling to dissolve the residue. (A small amount of completely insoluble material may occasionally remain.)

With urines from cases of Simmond's and Addison's disease, where a low ketosteroid content is expected, and with children's urines, only 2 ml. (or 1 ml.) of alcohol should be used. This alcohol extract is stable and may be preserved until it is convenient to complete the analysis.

Test. 0.2 ml. of the alcohol extract.

Standard. 0.2 ml. of the androsterone standard solution (≡0.1 mg. androsterone).

Blank. 0.2 ml. of alcohol.

Colorimetric Estimation. 0.2 ml. of the alcoholic solution of dinitrobenzene and 0.2 ml. of alcoholic 2.5 N-potassium hydroxide are added to the test, standard and blank. The tubes are stoppered and placed in a beaker of water at 25° for 1 hour in a dark place. 10 ml. of absolute alcohol are added to each of the tubes and the contents mixed.

The colour produced by the 17-ketosteroid is often complicated by the presence of a colour developed from other substances in the extract from the urine. This can be corrected for by reading the colours first in green light and then in violet ; the ratio of the intensity of the colours being different for the ketosteroid and the other substances when read with a green and a violet light filter. With the photoelectric colorimeter (or the M.R.C. photometer or the Duboscq used as a photometer) the test and blanks are read with the violet (621) and then with the green (624) light filters setting the

zero with the blank. The standard is read only with the green filter.

CALCULATION

Talbot, Berman and MacLachlan (1942) have found that the test reading (extinction) in the green light may be corrected by subtracting 0.6 of the reading (extinction) in the violet, and dividing the result by 0.73. The reading so corrected can then be compared directly with the reading of the standard in green light for calculation of the concentration.

$$\text{17-Ketosteroid}^{*} = \frac{\text{Reading of test green} - (0.6 \times \text{Reading of test violet})}{0.73 \times \text{Reading of standard green}} \times 0.1 \times \frac{4}{0.2}\,\dagger$$

* in mg. per 100 ml. urine.

† or 2 where 2 ml. of alcoholic extract have been used. The result should be expressed in mg. per twenty-four hours.

SOLUTIONS

Hydrochloric Acid. Pure, concentrated, Analar.

Carbon Tetrachloride. Analar.

Absolute Alcohol. The purity of the alcohol is important. The very pure Absolute Alcohol R.R. grade of the Distillers' Co. Ltd., A.D. Department, 21 St. James's Square, London, S.W.1, has been satisfactory without further purification.

Other alcohols may be purified as follows : to 1 litre of alcohol are added 4 g. of *n*-phenylenediamine hydrochloride. The mixture is allowed to stand in a dark place with occasional shaking for a week ; when it is distilled, the first and last 200 ml. of distillate are discarded and the middle fraction kept for use.

2 N-*Sodium Hydroxide* (approx.). 80 g. of Analar sodium hydroxide are dissolved in water and made to 1 litre.

Androsterone Standard. 50 mg. of androsterone (or of dehydro-*iso*androsterone) are dissolved in 100 ml. of the purified alcohol. 0.2 ml. of this standard solution contains 0.1 mg. of androsterone.

m-*Dinitrobenzene.* Purified *m*-dinitrobenzene can be obtained from most chemical supply houses, e.g. B.D.H., but it is necessary to purify it further by the following means : to 750 ml. of 95 per cent alcohol in a 4–5 litre flask are added 20 g. of *m*-dinitrobenzene, warmed to 40°C. and 2 N-sodium hydroxide added (about 100 ml.) until no further pink colour appears. The solution is well mixed and cooled after 5 minutes. 2.5 litres of water are added and well

mixed. The precipitated *m*-dinitrobenzene is collected by filtering on a Buchner funnel, where it is washed with several changes of distilled water and sucked dry. It is twice recrystallized from about 100 ml. of the purified absolute alcohol. If a proper degree of purity has been attained the crystals will be almost colourless needles of m.p. 91°C.

2 g. of the purified dinitrobenzene are dissolved in absolute alcohol in a total volume of 100 ml. This solution should be kept in a glass-stoppered brown bottle in the dark. It is stable for 2 weeks.

2.5 N-*Potassium Hydroxide* (approx.). 9 g. of Analar potassium hydroxide are dissolved in 50 ml. of the purified alcohol and the solution filtered through a hardened filter paper (Whatman 50). The concentration of this alcoholic potassium hydroxide is checked by titration against standard sulphuric acid (p. 186) and adjusted within the limits 2.48–2.52 N. It should be preserved in a refrigerator and should be discarded as soon as the faintest yellow colour develops.

Wilson and Carter (1947) have described the preparation of an alcoholic potassium hydroxide solution stabilized with ascorbic acid, which is stable up to 2 months.

Chapter VIII

ANALYSIS OF CALCULI

Calculi, although occasionally composed of a single constituent, are commonly mixtures of substances, and should always be cut or sawn to ascertain the presence or absence of different layers of deposits. The layers should be tested separately.

Renal Calculi. These most often contain carbonate, oxalate, calcium, uric acid or urates, ammonium salts and phosphates; more rarely cystine, 'urostealith' (fatty mixtures), fibrin and xanthine.

Biliary Calculi. The following substances may be present in these stones: cholesterol, bile-pigments and calcium.

SCHEME OF TESTS

Renal Calculus

The calculus is powdered in a mortar (separate layers being treated separately). Some of the powder is treated in a test tube with cold N-nitric acid. Effervescence indicates *carbonate*. The solution is gently boiled, cooled, and filtered. Some of the filtrate is made alkaline with ammonia solution. A precipitate indicates *calcium oxalate* or *phosphate*, the phosphate precipitate being soluble in acetic acid (now added in excess), the oxalate insoluble. The presence of phosphates may be confirmed by addition of ammonium molybdate solution to the nitric acid mixture. A yellow precipitate on boiling, or an intense blue colour on the addition of amino-naphtholsulphonic acid reagent (cf. plasma phosphate, p. 68), confirms phosphate. The presence of oxalate may be confirmed by its conversion to carbonate by heating some of the precipitate to red heat over a flame and treating the residue with N-hydrochloric acid, when effervescence indicates *oxalate*. This solution is treated with ammonium oxalate solution and

neutralized with ammonia. A crystalline precipitate confirms *calcium*.

More of the powder is boiled in a test tube with N-sodium hydroxide; an odour of ammonia indicates *ammonium salts*. 1 ml. of the filtered solution is treated with about 0.3 ml. of Folin's uric acid reagent and then with 1 ml. of sodium cyanide-urea solution. A blue colour shows *urates* or *uric acid*. The presence of these compounds may be further tested by evaporating a mixture of the powder with a little concentrated nitric acid to dryness in a porcelain dish. A red colour, becoming reddish-violet on addition of excess ammonia, shows *urates* or *uric acid*—this is the 'murexide' reaction. If ammonium salts are present, the urate is probably present as ammonium urate.

Cystine burns with a pale blue flame. Its presence may be confirmed by boiling the sodium hydroxide solution of the powder with a little lead acetate: a black precipitate is given by cystine. Typical hexagonal crystals are formed if an ammoniacal cystine solution is allowed to evaporate.

Urostealith calculi are soluble in alcohol and ether. *Fibrin* burns with a 'burnt feather' odour and a yellow flame, and dissolves in hot N-potassium hydroxide, from which it is precipitated by acetic acid, with evolution of hydrogen sulphide.

Xanthine burns without a flame. It gives a yellow residue, turning orange with sodium hydroxide, in the murexide test.

Biliary Calculus

A little of the dry powder from a biliary calculus is well shaken with chloroform. A yellow colour, which turns red or blue on addition of the Ehrlich diazo reagent and excess of alcohol shows *bile-pigments*. 5 ml. of the filtered chloroform solution are treated with 2 ml. of acetic anhydride and 2 drops of concentrated sulphuric acid. A green colour shows *cholesterol*.

REAGENTS REQUIRED

Concentrated nitric and sulphuric acids; glacial acetic acid; acetic anhydride; alcohol; ether; chloroform; N-hydrochloric acid; N-ammonia; N-sodium and potassium hydroxides; lead

acetate ; ammonium molybdate ; ammonium oxalate ; sodium cyanide-urea ; Folin's uric acid reagent ; Ehrlich's diazo reagent [freshly made mixture of 10 ml. of solution 'A' (1 g. sulphanilic acid+250 ml. N-hydrochloric acid+water to 1 litre) and 0.3 ml. of solution 'B' (sodium nitrite, 0.5 g. per 100 ml.) (p. 39)].

SOLUTIONS

N-*Hydrochloric Acid.* 10 ml. concentrated acid diluted to 100 ml. with water.

N-*Nitric Acid.* 6.33 ml. concentrated acid diluted to 100 ml. with water.

N-*Acetic Acid.* 6 ml. glacial acetic acid diluted to 100 ml. with water.

N-*Ammonia Solution.* 5.7 ml. concentrated ammonia diluted to 100 ml. with water.

Ammonium Molybdate Solution. 5 g. ammonium molybdate dissolved in water and made to 100 ml. (see p. 68).

Aminonaphtholsulphonic Acid. See p. 68.

Ammonium Oxalate. Saturated (4 per cent) solution (see p. 84).

N-*Sodium Hydroxide.* 4 g. per 100 ml.

N-*Potassium Hydroxide.* 5.6 g. per 100 ml.

Folin's Uric Acid Reagent. See p. 14.

Sodium Cyanide-Urea Solution. See p. 14.

Lead Acetate. 10 g. in 100 ml. of water.

Chapter IX

GASTRIC AND DUODENAL ANALYSIS

GASTRIC TEST MEAL

PRINCIPLE

Test meals are tests of gastric function. Through them a study is made of the gastric secretion—of the quality and quantity of the gastric juice in relation to its different constituents. The presence of abnormal constituents is noted, and the time it takes the meal to leave the stomach. The factors influencing gastric secretion are psychic, humoral and chemical. Tests of function are based on the response to chemical and humoral stimuli. The chemical stimulus used is usually a simple meal of dry toast or oatmeal gruel, or of dilute alcohol. This type of stimulus may be called 'the physiological stimulus.' Humoral stimulus is obtained usually by injection of a small amount of histamine. This may be called 'the pharmacological stimulus.'

Histamine Test

Histamine has definite advantages over the ordinary test meal. It evokes a maximum response, and often produces acid secretion where the ordinary test meals fail completely. It is independent of the psychic factors involved in the taking of any ordinary meal. It adds nothing to the stomach so that a pure juice, undiluted and uncontaminated, is obtained; and no neutralization of the acid can take place through food constituents.

Combined Alcohol and Histamine Tests

Alcohol as a physiological stimulant has several advantages over gruel, charcoal biscuit or dry toast. It is much less objectionable to take, is easily swallowed, and evokes a feeling of pleasure instead of distaste on the part of the subject.

The gastric juice obtained is ideal for analysis. It is thin and easily pipetted. No suspended food material obscures the colour and renders it turbid. The presence of bile, mucus and altered blood are much easier to detect, and the end-points in the titration are sharper and better defined.

Alcohol seems to cause a more ready response of gastric secretion than the other test meals. The curve of acidity rises more sharply. There is not the preliminary drop following the resting specimen which is observed in most fractional test meals, and is probably due to the neutralization of the first juice secreted, by the food stuff administered. Alcohol does not neutralize any of the acid. It is a neutral substance and only dilutes the juice. Further, a sufficient amount can be given in much less volume so that the dilution factor is small. Instead of a pint of gruel, only 50 ml. of aqueous alcohol are usually given.

The response to alcohol is much more quickly over—partly because it does not remain so long in the stomach. The purely liquid meal is soon evacuated. Most alcohol test meals show a decline in acidity by the end of an hour. A test meal of suspended solids takes $1\frac{1}{2}$ to 2 hours.

Administration of histamine following the alcohol effect gives a test which combines the advantages of the simple physiological type of stimulus with those very definite ones possessed by the pharmacological type of stimulus; and it allows of comparing in the same subject the two types of response.

The combined test (which is really two tests in one) is quicker than an ordinary test meal and yields more valuable information.

METHOD OF THE COMBINED TEST

The patient is given a light supper the night previous. The tube is passed and the stomach emptied as far as possible of its resting juice. 50 ml. of 7 per cent alcohol are then given the patient to drink. About 10 ml. of fluid is withdrawn at the end of 15, 30, 45 minutes and 1 hour. 0.5 mg. of histamine is then given by intramuscular injection. Samples

of juice are taken at ½ and 1 hour. The total time consumed by the test is thus 2 hours.

Resting juice should be studied in relation to its volume and nature. Attention should be paid to the presence of a foul odour and to the presence of abnormal constituents such as altered blood and lactic acid.

The *test meal samples* of juice should be studied particularly in regard to their free and total acidity. The passage of fluid from stomach to duodenum—and hence the condition of the pylorus, is deduced from the shape of the curve. Regurgitation from the duodenum is looked for in the presence of bile.

INTERPRETATION

Gastric Carcinoma. There is usually an absence of free HCl during the alcohol portion of the test. Histamine may, or may not, confirm this achlorhydria. The combined acid usually turns out to be organic acid ; a strong test for lactic acid is found. Blood is frequently present, and mucus is excessive. The foul smell of the resting juice is characteristic.

Gastric Ulcer. The results are very irregular, but are usually fairly like the normal. The curves for free and total HCl are in many cases within the normal range, although high results are often encountered. Mucus and bile may or may not be present. The presence of altered blood—'coffee-grounds'—is significant.

Stenosis is evidenced by an increasing acidity which does not show the normal tendency to fall, giving a 'plateau' type of curve. The presence of blood with an increasing acidity curve indicates a gastric rather than a duodenal ulcer.

No differentiation can be made on the basis of the curve between a simple hyperchlorhydria and one due to an organic lesion. Only the presence of blood will indicate the latter.

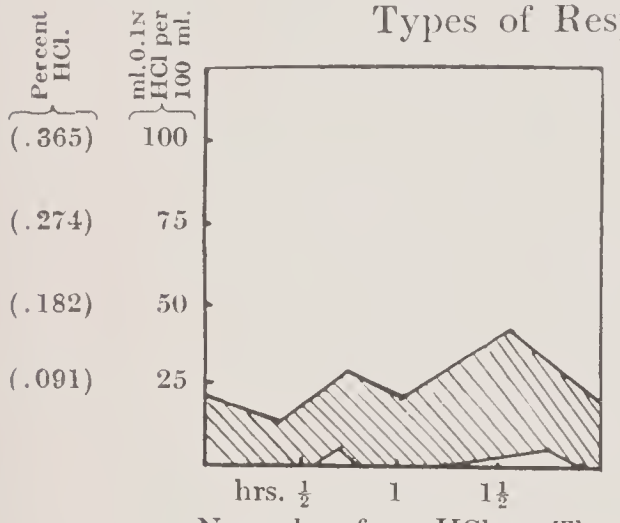
Duodenal Ulcer. Hyperchlorhydria is usually present : it may be very marked. If the ulcer be just below the pylorus, stenosis may result. The acid curve will then be very high and will not fall for some time. Other findings are not of note.

Achlorhydria. True achlorhydria is found in pernicious anæmia. No secretion is brought out by either the alcohol or the histamine.

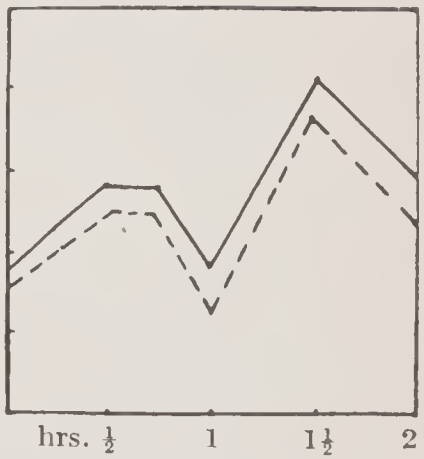
Apparent Achlorhydria, on the other hand, shows an absence of free HCl in the alcohol part of the test, but the histamine successfully stimulates its secretion.

FRACTIONAL TEST MEALS

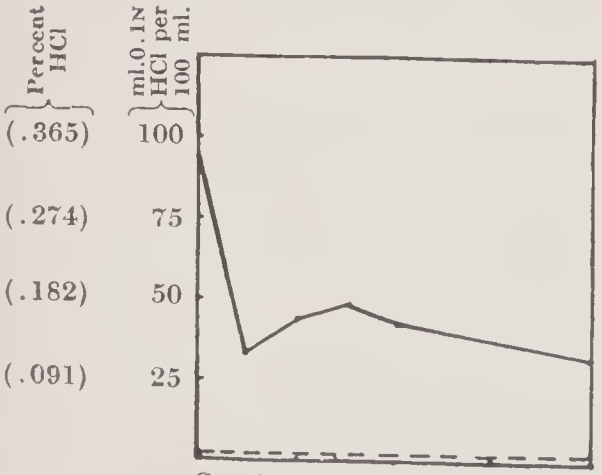
Alcohol and Histamine : Types of Response



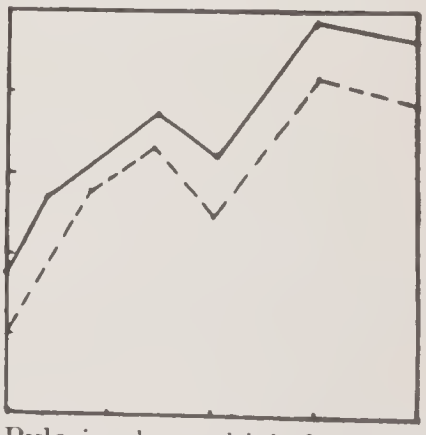
Normal : free HCl. The area within which most values fall with normal persons is shaded.



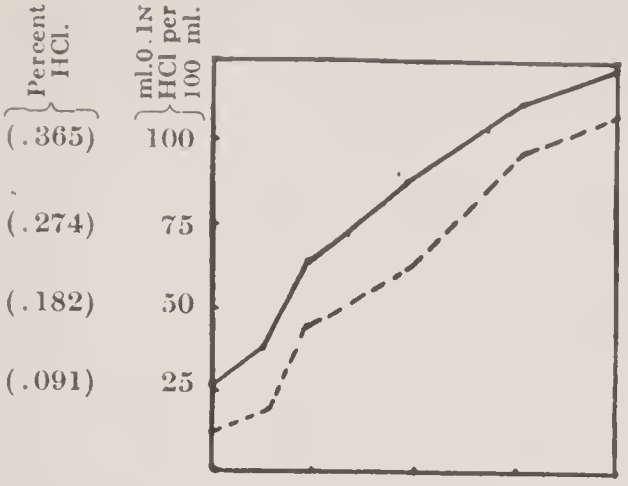
Gastric ulcer : fairly high free and total HCl ; small amount of mucus ; no bile or blood. Total acid, — ; free HCl, - - -.



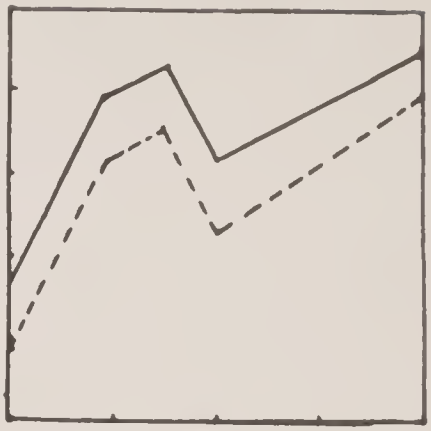
Carcinoma : resting juice of foul odour ; blood present ; no free HCl, high total (probably organic), lactic acid and mucus present.



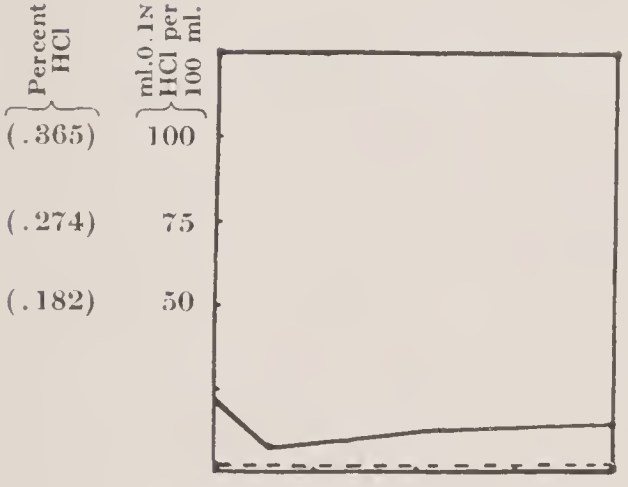
Pyloric ulcer : high free and total HCl ; no starch or mucus ; bile present ; no blood ; no lactic acid.



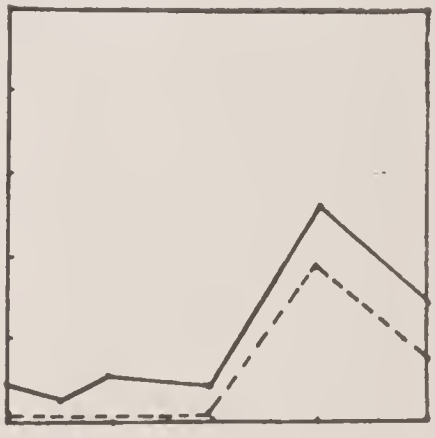
Duodenal ulcer; resting juice normal: marked response to stimuli in free and total HCl; slow emptying (plateau type); bile present, no blood.



Duodenal ulcer; free and total HCl rather high; no mucus, bile or blood.



Pernicious anæmia: no free HCl; low total; no starch; mucus and small amount of bile present.



Apparent achlorhydria (gastric ulcer); little mucus and bile; no blood.

Fig. 8.

Gastro-enterostomy, if successful, relieves hyperchlorhydria completely. The free HCl is very low or absent, due to the constant regurgitation of bile. The curve of total acidity is a low normal.

Partial Gastrectomy. The curve of free acidity is low. Bile is frequently present. The stomach empties quickly. If the operation has been unsuccessful the acidity curves will continue to be high. A routine test meal should be conducted within a few weeks of the operation.

ANALYSIS OF GASTRIC CONTENTS

Gastric Acidity. 5 ml. of the stomach contents are pipetted into a porcelain dish containing 2 to 3 drops of a mixture of equal volumes of Töpfer* and phenolphthalein indicators (p. 191).

The mixture is titrated with N/20 sodium hydroxide until a permanent orange colour is obtained (the point at which all the free acid is neutralized). Titration is then continued until the colour of the solution becomes red (total acid).

The results are multiplied by ten and thus expressed as ml. of N/10 sodium hydroxide required to neutralize 100 ml. of stomach contents.

Bile. A few ml. of the stomach contents are put into a test tube and a few drops of N/50 iodine added. A green colour denotes bile.

Mucus. Mucus is detected by the stringy appearance of the stomach contents.

Blood. Blood is detected in the same manner as 'occult blood' in fæces (see p. 103).

Lactic Acid. 2 ml. of stomach contents in a test tube are shaken with 5 ml. of ether. The mixture is allowed to separate and the ether layer is removed to another tube.

The ether layer is evaporated off by immersing the tube in hot water.

The residue is dissolved in 2 ml. of water and a few drops of MacLean's reagent are added. A yellow colour indicates the presence of lactic acid.

MacLean's Reagent is a mixture of:—
100 ml. saturated mercuric chloride solution (5 per cent.)
1.5 ml. concentrated hydrochloric acid
5 g. ferric chloride

TOTAL CHLORIDE IN GASTRIC JUICE

Volhard Method

5 ml. of gastric juice are pipetted into a 25 ml. volumetric flask and 10 ml. of N/5.85 silver nitrate in nitric acid solution

* Töpfer's indicator is a solution (0.5 g. per 100 ml.) of dimethyl yellow in alcohol.

(as used for the urine method) are added. The solutions are well mixed, made to the mark with water and again mixed. After 10 minutes the mixture is filtered. 10 ml. of the clear filtrate (≡2 ml. of gastric juice and 4 ml. of silver nitrate) are diluted with water in a porcelain dish, 2 ml. of ferric alum added, and titrated with N/5.85 ammonium thiocyanate solution to the final permanent pink colour.

CALCULATION

1 ml. N/5.85 silver nitrate = 10 mg. NaCl.

$\therefore$ mg. NaCl in 2 ml. gastric juice $= (4 - \text{titre}) \times 10$

$\therefore$ mg. NaCl in 100 ml. gastric juice $= (4 - \text{titre}) \times 10 \times \frac{100}{2}$

$= (4 - \text{titre}) \times 500$

PROTEOLYTIC ACTIVITY OF DUODENAL JUICE

Measurement of the concentration of the proteolytic enzymes, e.g. trypsin, in the duodenal juice is often of use in the study of pancreatic dysfunction, particularly in children. Incomplete digestion of protein may be suspected from examination of the fæces; but it is more satisfactory to determine the actual enzyme content of a sample of the aspirated juice. In pancreatic insufficiency the concentration of the enzyme, i.e. the proteolytic activity, may be considerably reduced.

PRINCIPLE

The principal protein-digesting enzyme in the juice of the duodenum is trypsin, which is secreted there by the pancreas. It is usual to estimate the content of proteolytic enzyme by studying the rate at which it digests (hydrolyses) casein. The amount of hydrolysis products formed from casein in a given time is taken as a measure of the amount of the enzyme present. The hydrolysis may be followed by determining the amount of liberated peptides or amino-acids in terms of the increase of titratable carboxyl or amino-groups. Another method is to determine the amount of residual casein precipitable by trichloroacetic acid after the digestion has taken place.

In the method given here, which is due to Charney and Tomarelli (1947), the chromophoric protein substrate, sulphanilamide azocasein, is used for the determination of the proteolytic activity. This substance is precipitated by trichloroacetic acid, but its potentially coloured digestion products remain in solution. When the enzyme action is stopped the residual undigested azocasein is precipitated by the addition of trichloroacetic acid. The amount of colour in the filtrate can then be taken as the measure of the extent of the hydrolysis, and thence of the enzyme concentration.

METHOD

A specimen of duodenal juice is centrifuged for about 10 minutes, and 1 ml. is withdrawn with a pipette from the middle layer, which is usually the least turbid part of a centrifuged specimen. The 1 ml. sample is diluted to 100 ml. with sodium bicarbonate buffer.

Test. 1 ml. of the buffer-substrate solution is put in a test tube and placed in a 37°C. water bath. Some of the diluted duodenal juice is placed in another tube and this is likewise placed in the 37°C. water bath. After about 3 minutes, when both solutions have reached 37°C., 1 ml. of the diluted duodenal juice (≡0.01 ml. of original juice) is transferred to the tube containing the substrate, with which it is mixed. The digestion is allowed to proceed for 30 minutes, when 8 ml. of 5 per cent trichloroacetic acid are added to stop digestion and to precipitate the remaining azocasein. The contents of the tube are well mixed and filtered.

Blank. A substrate blank is made consisting of 1 ml. of the buffered substrate and 1 ml. of bicarbonate buffer in place of the diluted duodenal juice. This mixture is likewise treated with 8 ml. of 5 per cent trichloroacetic acid and filtered.

To 5 ml. of the filtrate from each of the test and blank are added 5 ml. of N/2-sodium hydroxide. The intensities of the resulting orange-red colours are determined in a photoelectric or visual colorimeter, and the results expressed as extinctions. The reading (extinction) of the blank is subtracted from that of the test. Since the red colour obeys

Beer's Law the extinction may be taken as a direct measure of the amount of hydrolysis, and hence of the enzyme activity.

Standard. In case it may be desired to express the result in terms of the percentage of digestion of the azocasein which has taken place, the colour of the test (corrected by subtracting the blank) may be compared with the colour of the following standard :—

A 1 in 25 dilution is made of the buffer substrate (4.0 ml. of the buffered substrate diluted to 100 ml. with sodium bicarbonate buffer). 5 ml. of this standard diluted substrate is treated with 5 ml. of N/2-sodium hydroxide. The full orange-red colour produced is due to the azocasein at the 1 in 25 dilution.

The violet light filter Ilford 621 is used for the measurement of the colours.

CALCULATION

The result may be expressed simply in terms of the extinction of the test corrected by the subtraction of the blank or as a percentage of the azocasein digested (see above).

Photoelectric Colorimeter.

$$\text{Proteolytic activity}^* = \frac{\text{Reading of test} - \text{Reading of blank}}{\text{Reading of standard} \times 25} \times \frac{100}{0.01}$$

* Calculated as the percentage hydrolysis of the azocasein which would have resulted if 1 ml. of duodenal juice had been used instead of the 0.01 ml. (1 ml. of the 1 in 100 dilution) actually taken.

SOLUTIONS

Sulphanilamide-Azocasein. 5 g. of reprecipitated fat-free casein is dissolved in 1 litre of distilled water with 10 g. of sodium bicarbonate in a 2-litre beaker. In another beaker (250 ml.) 5 g. of sulphanilamide are dissolved in 200 ml. of distilled water with 3 ml. of 10 N-sodium hydroxide (40 per cent, p. 187). 2.2 g. of sodium nitrite ($NaNO_2$) are added and stirred into solution. 9 ml. of concentrated hydrochloric acid (10 N) are now added and the mixture stirred for 2 minutes. 9 ml. of 40 per cent sodium hydroxide are added, well stirred, and the mixture added at once to the solution of casein.

The mixture is acidified and the sulphanilamide-azocasein precipitated by the addition of hydrochloric acid until acid to

Congo red paper. The precipitate is filtered on a Büchner funnel, washed with water and alcohol, and dried by spreading on filter paper at room temperature. The azocasein so obtained is an orange-red material which should be powdered and stored in a brown bottle.

Buffered Substrate. 2.5 g. of the sulphanilamide-azocasein and 0.5 g. of sodium bicarbonate are dissolved in water in a 100 ml. volumetric flask by shaking and warming under the hot water tap. The solution is adjusted to the mark, well mixed and kept in a refrigerator. The pH should be tested and should be 8.3.

Buffer. A 0.5 per cent solution of sodium bicarbonate.

Chapter X

TESTS OF FUNCTION

THE GLUCOSE TOLERANCE TEST

The patient is fasted for 12 hours or longer. The 'fasting blood sugar' is estimated by the method described (p. 20). Immediately after the blood has been taken, a solution of 50 g. of glucose in 250 ml. of water is given (for youthful subjects,

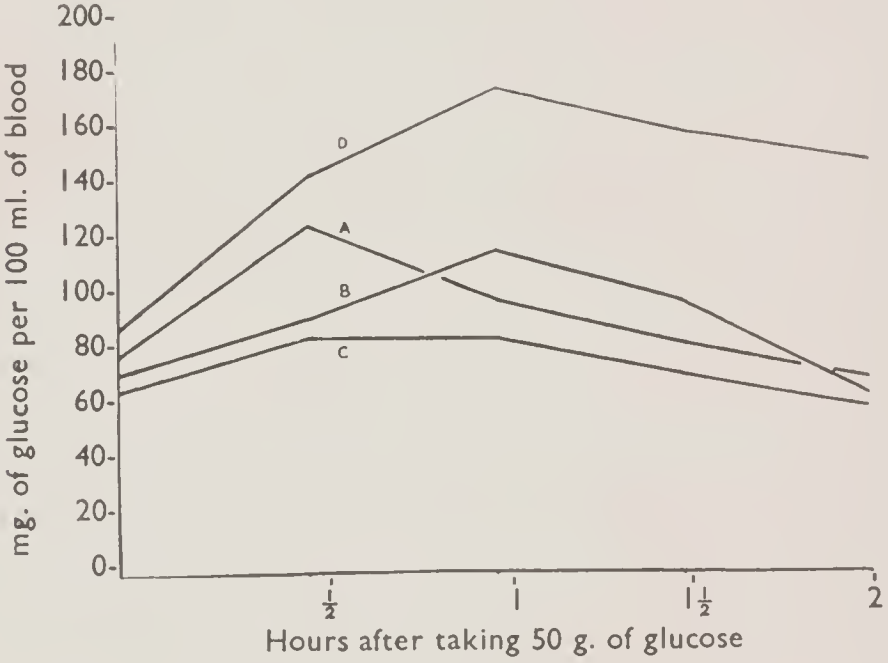


Fig. 9. Glucose Tolerance Tests.

A & B. Normal.
C. Increased tolerance (e.g. Addison's disease).
D. Decreased tolerance (Diabetes).

the amount of glucose should be 1 g. per 3 lb. of body weight). Blood sugar estimations are then made at ½, 1, 1½ and 2 hourly intervals after the administration of the glucose. Samples of urine are taken before the test and 2–4 times during its course. In normal persons, the blood sugar will not generally rise above 160 mg. per 100 ml., and the urine will not give a positive test for sugar. The blood glucose level

will show a sharp peak after ½ to 1 hour, falling to or nearly to normal at the end of 2 hours. In diabetic cases, the blood glucose may rise to very high levels (300–600 mg. per 100 ml.) and *only returns very gradually to normal.* Sugar is almost always present in the urine.

INSULIN-GLUCOSE TOLERANCE TEST

In adrenal and pituitary insufficiency and also in hyperinsulinism there is an exaggerated response to insulin, a spontaneous rise in the level of the blood sugar after hypoglycæmia being retarded. Fraser, Albright and Smith (1941) described an insulin-tolerance test, and a glucose-insulin tolerance test. Engel and Scott (1950) have described a modified glucose-insulin-tolerance test in which 0.1 unit of insulin per kg. of body-weight is injected intravenously in the fasting subject. Samples of blood are taken before the injection, and at 30 minutes (or before if symptoms of hypoglycæmia appear). Glucose (0.8 g. per kg. body weight) is given by mouth immediately following the taking of the 30-minute specimen of blood. Further blood specimens are taken at 1 hour, 1½ hours, 2 hours and 3 hours after the injection of the insulin.

It is convenient to express the results in percentages of the initial blood sugar value. The average response of normal individuals is as follows : at 30 minutes blood sugar is 45 per cent of the initial value ; at 1 hour it is rising sharply, and at 1½ hours it is 150 per cent. (For example, if the initial blood sugar were 100 mg. per 100 ml. the average value at 30 minutes would be 45 mg. and that at 1½ hours would be 150 mg.) At 2 hours the blood sugar has shown a distinct fall and at 3 hours it has fallen to a value slightly below that of the initial level. In cases suffering from Addison's disease there is a decrease in response to glucose after insulin, which results in the production of curves which are relatively flat compared to the normal. Cases of Simmonds' disease yield similar results. In Cushing's disease and in acromegaly the opposite type of response is elicited : there is an impaired fall in blood sugar after insulin with either, or both, an

exaggerated rise and a delayed fall in the blood sugar after the administration of glucose.

GALACTOSE TOLERANCE TEST

Transformation of Non-glucose Sugars into Glycogen

Galactose, in common with various other sugars, e.g. lævulose, xylose, is not metabolized directly in the body as is the case with glucose, but must first be transformed into glycogen. The capacity of the liver for transforming these other sugars into glycogen has been found to be altered in certain conditions of impaired liver function, and the rate of removal of the sugar in question from the blood forms the basis of an estimate of the capacity of the liver for transforming the non-glucose sugar.

By the galactose tolerance test it is possible only to detect fairly severe degrees of liver damage. The test, in conjunction with other investigations (e.g. bilirubin and phosphatase determinations) may be useful in differentiating between obstructive and non-obstructive jaundice (King and Aitken, 1940).

Galactose tolerance has been used by Barnes and King (1942) as a test for thyrotoxicosis. Blood galactose rises to high values and fails to return to normal in two hours (a diabetes-like curve).

MODE OF ADMINISTRATION

As most commonly employed, the test consists in the oral administration of 40 g. of galactose, followed by analysis of the urine passed in the succeeding five hours to determine the total amount of galactose excreted. If the liver (and to a lesser extent, other tissues) has metabolized the administered galactose to such an extent that less than 3 g. is excreted in the five hours following ingestion, its functional capacity is held to be unimpaired. The excretion of more than 3 g. of galactose is considered to indicate subnormal hepatic function.

A more exact procedure is to estimate the rate of disappearance of galactose from the blood. The galactose may be given orally or intravenously.

EXPERIMENTAL PROCEDURE

A solution of galactose (50 g. per 100 ml. of solution) is prepared and sterilized by filtration and steaming.

Oral Galactose. The patient receives no breakfast. An amount of solution equivalent to 40 g. of galactose (80 ml. of the 50 per cent solution), or 40 g. of the solid, suitably diluted with water, is given the patient to drink.

Intravenous Galactose. An injection of 50 ml. of a 50 per cent solution of galactose is given slowly over about 5 minutes into an arm vein.

Samples of Blood. The first sample of blood is taken immediately before the administration of the galactose (resting sample), the second sample at $\frac{1}{2}$ hour, and further samples at 1, $1\frac{1}{2}$, and 2 hours. The resting sample is a useful check on the analytical method because it should contain no galactose.

Analysis. The samples of blood (0.2 ml. of capillary blood) are washed into centrifuge tubes containing isotonic sodium sulphate and sodium tungstate. The estimation is carried out as described on p. 26.

RESULTS

The galactose values are plotted against time. Both the oral and intravenous tests, for a normal person, usually give curves which begin at 0, rising to about 40 to 70 mg. of galactose per 100 ml. of blood at 30 minutes, fall steeply during the next hour, and reach a figure between 0 and 10 mg. per 100 ml. at the end of two hours. In most cases of obstructive jaundice the curve of blood galactose follows the same course, there being little or no galactose left in the blood at the end of two hours. In conditions of liver damage the level of galactose in the blood does not return to the normal level within the two hours.

In moderate thyrotoxicosis the blood galactose concentration following oral administration is moderately raised, and in severe thyrotoxicosis it is greatly elevated.

A useful method of expressing the results of the oral galactose test is the 'galactose index,' the sum of the blood

galactose values at $\frac{1}{2}$, 1, $1\frac{1}{2}$ and 2 hours. For normal subjects this averages about 60 and rarely exceeds 120. Patients with hepatic dysfunction, or thyrotoxicosis, may have greatly elevated indices, up to or over 500 (Maclagan, 1940 ; Barnes and King, 1942).

The 'galactose time' is a convenient way of expressing the results of the intravenous test. $\text{G.T.} = \frac{a}{a-b} \times 90$, where G.T. is the galactose time, 90 the minutes between the $\frac{1}{2}$-hour and 2-hour blood galactose values, a the value for blood galactose at $\frac{1}{2}$ hour and b that at 2 hours. In normal subjects the 'galactose time' is between 30 and 90, and may be elevated (up to 184) in severe hepatitis and cirrhosis (Sherlock, 1946).

INTRAVENOUS HIPPURIC ACID TEST

PRINCIPLE

The detoxicating power of the liver is assessed by administering benzoic acid (as the sodium salt) which is conjugated with glycine, in the liver, to form hippuric acid, and excreted as such by the kidneys. The specificity of the test for hepatic as opposed to renal function is not certain, and a simultaneous urea clearance test should be run.

METHOD

Administration. 20 ml. of an 8.85 per cent solution of sodium benzoate, =1.77 g. sodium benzoate, equivalent to 1.5 g. benzoic acid, is injected slowly into an arm vein. Immediately after the injection, and exactly one hour later, the bladder is emptied. The first specimen is discarded, and the hourly specimen used for the analysis. In order to ensure an adequate urine output, the patient is given a pint of water to drink after the injection.

Estimation. (Weichselbaum and Probstein, 1939). The urine volume is measured, and if it exceeds 120 ml. is acidified with a little glacial acetic acid, evaporated down and again measured. If the urine is very heavily pigmented, a little

charcoal is added. The warm urine is then saturated with salt, 30 g. per 100 ml., heated and filtered. The filtrate is acidified with 50 per cent sulphuric acid, till acid to Congo red and is left to stand in the cold, preferably overnight, until crystallization is complete. Crystallization can sometimes be induced by scratching the sides of the vessel with a glass rod, or by adding a small crystal of hippuric acid. The crystals are then filtered off by suction, washed with cold 30 per cent sodium chloride and dissolved in distilled water by warming. The solution is titrated against 0.5 N-sodium hydroxide, using phenolphthalein as indicator.

CALCULATION

Allowance must be made for the solubility of hippuric acid in urine saturated with salt at room temperature (0.123 g. per 100 ml.≡0.1 g. sodium benzoate).

1.0 ml. 0.5 N-NaOH≡0.072 g. sodium benzoate (C_6H_5COONa)

∴ Weight of hippuric acid excreted*

$$= \text{ml. NaOH used} \times 0.072 + \left(0.1 \times \frac{\text{Vol. urine}}{100}\right).$$

* Expressed as g. sodium benzoate.

Normal range (Quick, Ottenstein and Weltchek, 1938):—

0.86–1.12 g. expressed as sodium benzoate.
0.70–0.95 g. ,, ,, benzoic acid.

OTHER LIVER FUNCTION TESTS

Bromsulphthalein Test, see p. 92.
Colloidal Gold Reaction, see p. 90.
Thymol Turbidity Test, see p. 91.

UREA CLEARANCE TEST

The 'urea clearance test' (devised by Moeller, McIntosh and Van Slyke) aims to obtain a measure of the efficiency

with which the kidney excretes urea. It shows the response of the kidney to the stimulation of the actual amount of urea in the blood. The clearance is defined in terms of the number of millilitres of blood 'cleared' of urea per minute.

It has been found that with large volumes of urine (above 2 ml. per minute) the output of urea depends upon, and is directly proportional to the level of urea in the blood. Where the rate of urine excretion falls below 2 ml. per minute this direct relationship no longer holds and the output of urea is now found to vary, not only with the blood urea but also with the square root of the volume of urine.

In order to obtain a maximum physiological stimulation of the kidney, urea is given to the patient with a liberal drink of water before commencing the test. In cases where the blood urea is already high the water alone is given.

The necessary data are the urea concentration of the blood and of the urine, and the volume of urine excreted in the given time.

The normal 'maximum clearance' (when the flow of urine is above 2 ml. per minute) is usually about 40 per cent greater than the 'standard' clearance (flow of urine less than 2 ml. per minute). The average normal values are as follows :—

> For the 'maximum clearance' an average of 75 ml. of blood per minute with variations from 64 to 99 ml., and for the 'standard clearance' an average of 54 ml. with variation from 40 to 68 ml.

Nephritic patients with diminishing renal efficiency show a decreased 'clearance' (down to about 40 per cent) before the blood urea and the blood creatinine begin to rise ; on the other hand, the maximum specific gravity of the urine is often diminished before the urea clearance test gives abnormal results. When the clearance falls to 5 per cent the symptoms of uræmia usually appear.

METHOD

The patient is given no breakfast and nothing to drink in the early morning other than water if desired.

The test is carried out in the forenoon as follows :—

0 hours :	Empty bladder Give 15 grams urea in 500 ml. water, if the blood urea is not raised. (If blood urea is known to be raised, or likely to be raised, give 500 ml. water only.)*		No.
½ hour :	Take blood for urea estimation . .	Blood	1
1 hour :	Empty bladder†—send complete specimen to laboratory . . .	Urine	1
1½ hours :	Take blood for urea estimation .	Blood	2
2 hours :	Empty bladder†—send complete specimen to laboratory . . .	Urine	2

NOTE. The success of the test depends on collecting for analysis *all* the urine which has been excreted into the bladder during the stated time interval. If it is suspected that the patient's bladder is not being completely emptied, recourse should be had to catheterization. This is particularly important with hospitalized patients in bed.

If the excretion of urine is at a rate greater than 2 ml. per minute, the so-called 'maximum urea clearance' is calculated according to the following formula :—

$$C_m = \frac{UV}{B}$$

where C_m = maximum urea clearance, i.e. volume of blood 'cleared' of urea in 1 minute during moderate diuresis.

U = concentration of urea in urine (mg. per 100 ml.).

V = volume of urine in ml. per minute.

B = blood urea (mg. per 100 ml.).

* The patient's blood urea should usually be determined a day or two before the Clearance Test is carried out.

† The specimens of urine must be taken exactly at the times specified. If for any reason it is found necessary to deviate from the sixty-minute periods, the exact time (in minutes) during which the urine has collected (i.e. since the previous evacuation) should be specified.

If the excretion of urine is at a rate less than 2 ml. per minute, the so-called 'standard urea clearance' is calculated from the following formula :—

$$C_s = \frac{U}{B}\sqrt{V}$$

where C_s = standard urea clearance,

and U, V, and B are the same as above.

The result of the test is usually expressed as a percentage of the average normal clearance, which is taken to be 75 ml. of the blood per minute if the rate of urine excretion is above 2 ml. per minute (C_m), and 54 ml. blood if the rate is below 2 ml. per minute (C_s).

C_m is therefore multiplied by $\frac{100}{75}$ or 1.33,

and C_s by $\frac{100}{54}$ or 1.85.

Normal kidneys show a fairly wide variation in clearance (from over 100 to 70), but figures below 70 per cent usually indicate impaired renal efficiency. Examples are given below.

Urea Clearance Test on Normal Individual

	1st hour	2nd hour	3rd hour
Blood Urea	65 mg. per 100 ml.	75 mg. per 100 ml.	71.4 mg. per 100 ml.
Urine Urea	1950 ,, ,,	3000 ,, ,,	3930 ,, ,,
Urine Volume	121 ml.	86.4 ml.	35 ml.
Clearance	$C_m = \frac{1950}{65} \times \frac{121}{60}$	$C_s = \frac{3000}{75} \sqrt{\frac{86.4}{60}}$	$C_s = \frac{3930}{71.4} \sqrt{\frac{35}{60}}$
Blood cleared of Urea per minute	60.1 ml.	48 ml.	41.9 ml.
Per cent of Normal Function	$(C_m \times 1.33) = 80$	$(C_s \times 1.85) = 88.8$	$(C_s \times 1.85) = 77.4$

Urea Clearance Tests on Clinical Cases

1. (*m.* 19) *Nephritis*, blood urea before test 34 mg. per 100 ml. (15 g. urea in 500 ml. water administered) :—

	1st hour	2nd hour
Blood Urea	63.0 mg. per 100 ml.	69.0 mg. per 100 ml.
Urine Urea	1275 „ „	1770 „ „
Urine Volume	96 ml.	70 ml.
CLEARANCE	$C_s=\frac{1275}{63}\sqrt{\frac{96}{60}}$	$C_s=\frac{1770}{69}\sqrt{\frac{70}{60}}$
Blood cleared of Urea per minute	=25.6 ml.	=27.7 ml.
Per cent of Normal Function	$(C_s\times 1.85)=47$	$(C_s\times 1.85)=51$

Average=49 per cent of Normal Function.

2. (*f.* 18) *Subacute Nephritis*, blood urea before test 33.8 mg. per 100 ml. (15 g. urea in 500 ml. water administered) :—

Blood Urea	87.3 mg. per 100 ml.	102 mg. per 100 ml.
Urine Urea	900 „ „	895 „ „
Urine Volume	116 ml.	134 ml.
CLEARANCE	$C_s=\frac{900}{87.3}\sqrt{\frac{116}{60}}$	$C_m=\frac{895}{102}\times\frac{134}{60}$
Blood cleared of Urea per minute	=14.3 ml.	=19.6 ml.
Per cent of Normal Function	$(C_s\times 1.85)=27$	$(C_m\times 1.33)=26$

Average=26.5 per cent of Normal Function.

3. (*f.* 67) *Uræmia*, blood urea 193 mg. per 100 ml. (500 ml. water administered) :—

Blood Urea	209 mg. per 100 ml.	209 mg. per 100 ml.
Urine Urea	615 „ „	615 „ „
Urine Volume	100 ml.	95 ml.
CLEARANCE	$C_s=\frac{615}{209}\sqrt{\frac{100}{60}}$	$C_s=\frac{615}{209}\sqrt{\frac{95}{60}}$
Blood cleared of Urea per minute	=3.8 ml.	=3.7 ml.
Per cent of Normal Function	$(C_s\times 1.85)=7.0$	$(C_s\times 1.85)=6.9$

Average=6.95 per cent of Normal Function.

4. (*f.* 54) *Pyelonephritis, Uræmia*, blood urea 250, rising to 640 mg. per 100 ml., urine albumin 600 mg. per 100 ml.

Post Mortem : abscesses in both kidneys, subacute diffuse nephritis (500 ml. water administered) :—

	1st hour	2nd hour
Blood Urea	378 mg. per 100 ml.	250 mg. per 100 ml.
Urine Urea	902 ,, ,,	892 ,, ,,
Urine Volume	16 ml.	10 ml.
CLEARANCE	$C_s=\frac{902}{378}\sqrt{\frac{16}{60}}$	$C_s=\frac{892}{250}\sqrt{\frac{10}{60}}$
Blood cleared of Urea per minute	$=1.2$ ml.	$=1.5$ ml.
Per cent of Normal Function	$(C_s\times 1.85)=2.3$	$(C_s\times 1.85)=2.7$

Average$=2.5$ per cent of Normal Function.

DETERMINATION OF RENAL BLOOD FLOW AND GLOMERULAR FILTRATION RATE

PRINCIPLE

The renal plasma clearance (UV/P)* of a substance which is completely removed from the blood by the kidneys is equal to the renal plasma flow. No substance is consistently completely cleared, but at low plasma concentration of *para*-aminohippurate (PAH), its clearance is between 85 and 100 per cent of the renal plasma flow, and is known as the 'effective renal plasma flow' (ERPF). In the presence of gross tubule dysfunction PAH clearance is no longer a close approximation to renal plasma flow; and to determine the flow, the renal arterio-venous difference in PAH concentration must also be known (Bull, Jockes and Lowe, 1950).

It is convenient to combine with the ERPF determination the determination of glomerular filtration rate (GFR). The thiosulphate ion is filtered by the glomerulus and neither reabsorbed nor excreted by the tubules, and its clearance is equal to glomerular filtration (Gilman, Phillips and Koelle, 1946).

The fraction of plasma filtered at the glomerulus can be calculated as follows :—

$$\frac{\text{GFR}}{\text{ERPF}}=\text{Filtration Fraction (FF)}$$

*Where UV represents the amount of the substance excreted in a given time in the urine (U = its concentration in the urine and V the volume of urine), and P its concentration in the blood plasma.

The range of values in normal subjects of 1.73 sq. m. surface area* is as follows :—

	Male	*Female*
ERPF . . .	697 ±135.9	594 ±102.4
GFR . . .	131 ± 21.5	117 ± 15.6
FF . . .	0.19 ± 0.02	0.20 ± 0.03

Most kidney diseases are associated with low rates of ERPF and GFR, and these determinations provide the most accurate method of assessing the extent of kidney damage. The FF is characteristically raised in idiopathic hypertension and cardiac failure, and is low in acute nephritis. For other interpretations see Brun, Hilden and Raaschou (1949).

METHOD

Any sulphonamide administration is stopped two days before the test, as sulphonamides interfere with the estimation of PAH. Penicillin administration is stopped 24 hours before the test, as it interferes with the tubular excretion of PAH.

The patient is given no breakfast, and enough water to drink to ensure a urine flow of more than 2 ml. per minute. The test is carried out in the forenoon as follows :—

A sample of urine is collected (urine blank, U_0) and a 10 ml. sample of heparinized (p. 6) blood (blood blank, B_0).

The loading dose (10 g.) of thiosulphate is injected very slowly (over 10–15 minutes) intravenously, and the loading dose (1 g.) of PAH subcutaneously. (Some patients complain of nausea unless the thiosulphate injection is given very slowly.)

Approximately 30 minutes is allowed to pass for equilibration of the injected materials in the body. The bladder is catheterized, and is emptied by suprapubic pressure followed by the injection of approximately 100 ml. of air followed by approximately 50 ml. of water and then more air. When the last of the water has drained, the time is noted to the nearest $\frac{1}{2}$ minute—time R. The catheter is allowed to drain into a measuring cylinder ; and, when 50 ml. or more has collected the bladder is emptied again as above. The time S is noted.

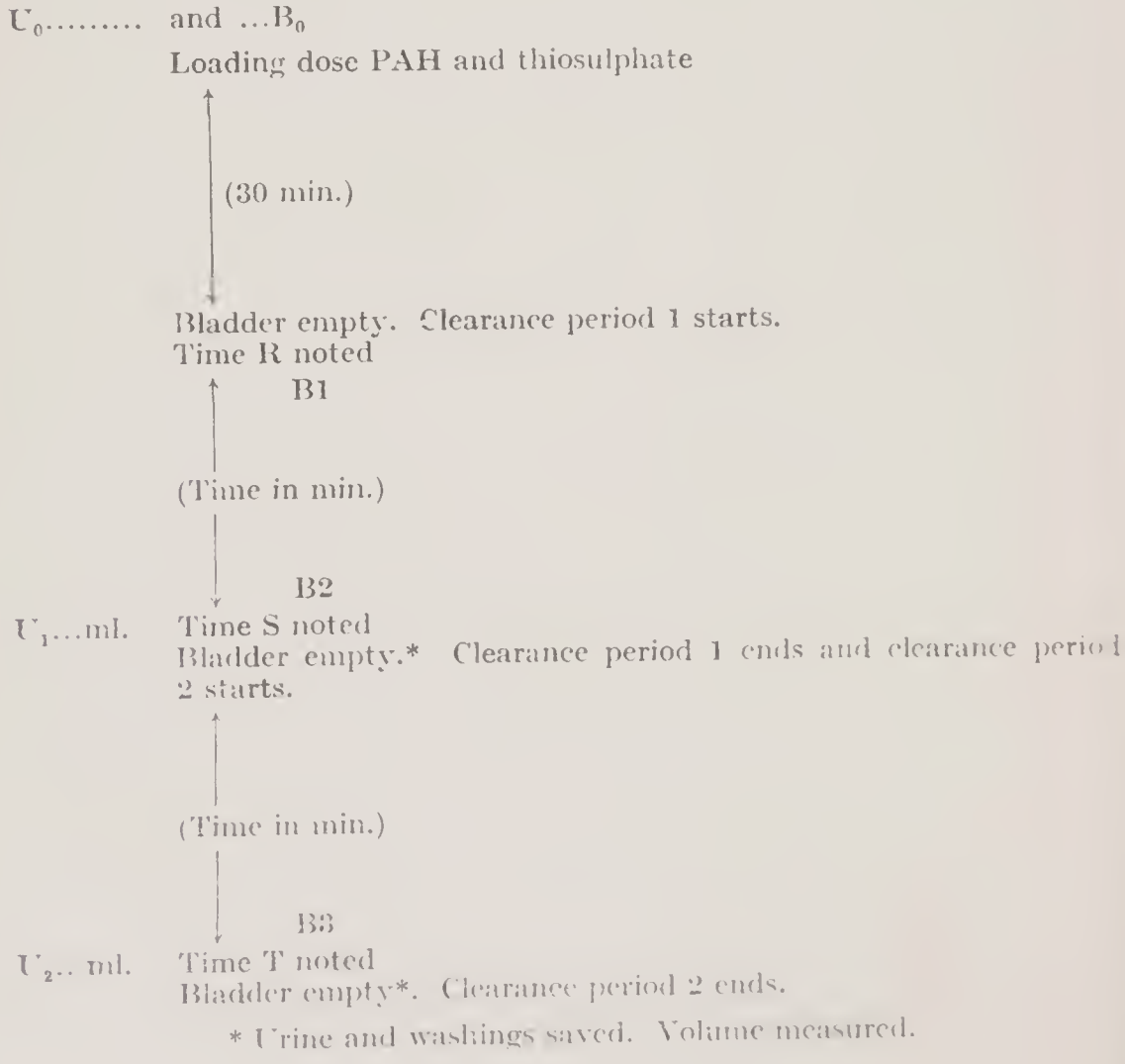
* Goldring & Chasis (1944) *Hypertension and Hypertensive Disease*, New York: The Commonwealth Fund.

The wash water is added to the collected urine and the whole mixed and measured, and a sample saved for chemical estimations (Urine U_1). This collection period is followed by a second. The time T is noted, and Urine 2 (U_2) is measured and a sample saved.

Approximately 10 ml. samples of heparinized blood are drawn at the beginning and end of the first period of collection, and at the end of the second period of collection (B1, B2 and B3).

The proportion of cells to plasma is determined in a hæmatocrit or graduated centrifuge tube on one of the blood samples ; and the plasmas are separated by centrifuging. The concentrations of PAH and thiosulphate are determined on all samples. (The PAH blank is unnecessary.)

The schedule of injections and collections is set out in the following diagram :—



* Urine and washings saved. Volume measured.

PARA-AMINOHIPPURATE (PAH) CLEARANCE

Estimation of PAH. This is carried out on the separated plasmas (B1, B2 and B3) according to the method for sulphonamides in blood on p. 27.* It should be noted that the dilution of the blood plasma with trichloroacetic acid is 1 in 20 in this procedure (see calculation).

The urines (U_1 and U_2) are diluted 1 in 200 to 1 in 1000, and are then treated in the same way as the trichloroacetic acid filtrates of blood,* the final dilution (usually the 1 in 1000) being taken into account in the calculation.

Calculation of PAH Clearances. It is unnecessary to determine the absolute concentrations of PAH as the ratios of colorimeter readings in urine and blood will give the result.

The relevant times and concentrations are plotted on graph paper as shown (Fig. 10). 2 minutes are subtracted from the midpoints of the clearance periods, and by intrapolation of the colorimeter readings at the times M and N the blood concentrations at these times (B_m and B_n) are estimated. This is to allow for the time taken for the urine to traverse the dead space of the renal pelves and ureters (Smith, Goldring and Chasis, 1938).

$$\text{Effective Renal Plasma Flow† (ERPF)} = \frac{\text{V (vol. urine + washings in ml.)}}{\text{T (time of urine collection in min.)}}$$

$$\times \frac{\text{U (colorimeter reading)} \times \text{dilution of urine}}{\text{2 (ml. dilute urine used)}}$$

$$\times \frac{\text{2 (ml. plasma filtrate used)}}{\text{B}_{(\text{m or n})} \times \text{20 (dilution of plasma)}}$$

$$= \frac{\text{V}}{\text{T}} \times \frac{\text{U} \times \text{dilution of urine}}{\text{B}_{(\text{m or n})} \times 20}$$

† in ml. plasma cleared per minute.

*With the addition of 1 ml. N-hydrochloric acid to the 2 ml. of trichloroacetic acid filtrate or diluted urine.

ERPF determined in this way is subject to a slight error resulting from falling or rising blood concentrations of PAH. The coefficient of variation using this technique is about 15

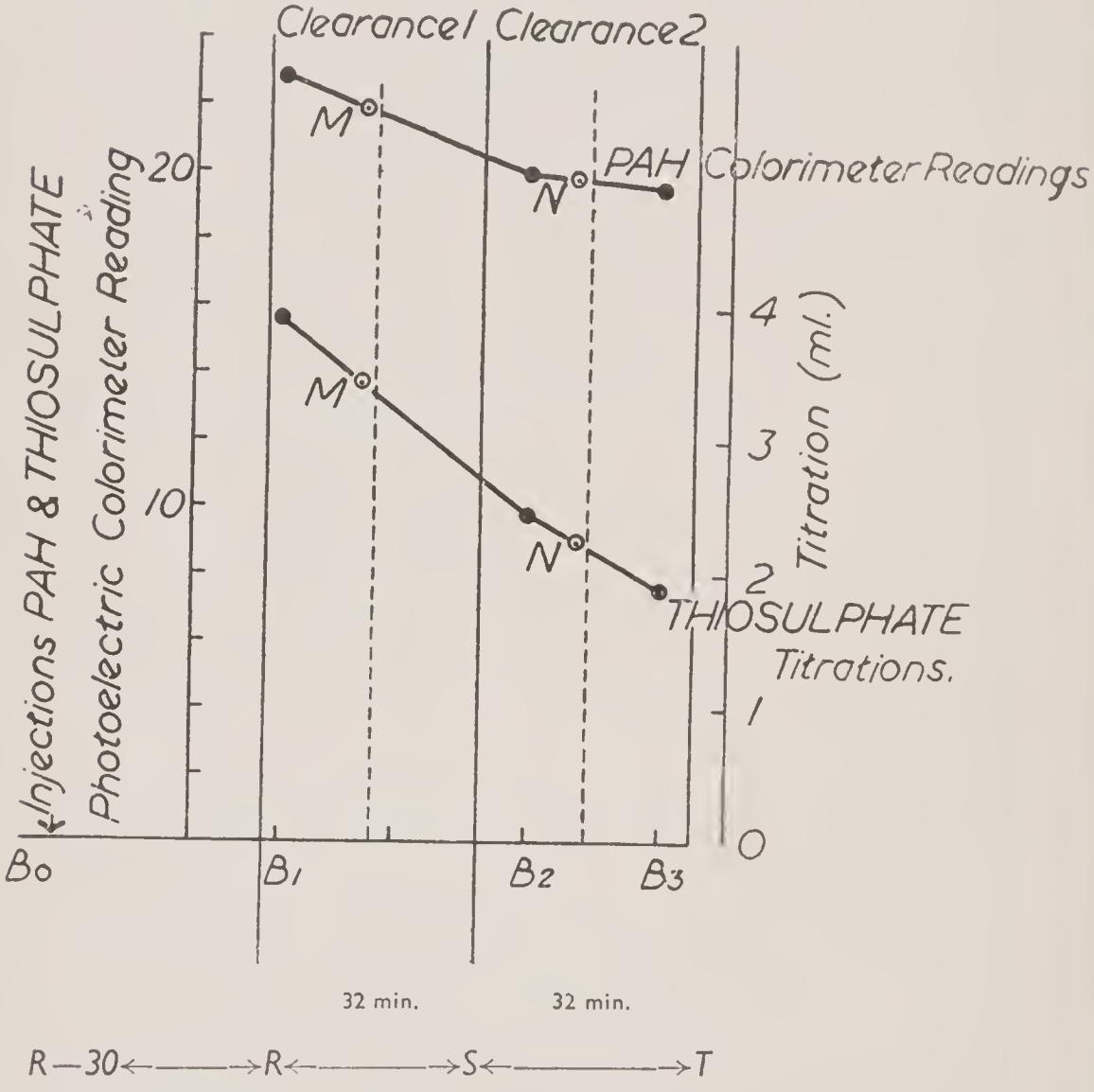


FIG. 10. Scheme for collection of blood and calculation of results for *p*-amino hippuric acid and thiosulphate clearance tests.

per cent. This error becomes progressively less with increasing degrees of renal damage. Where more accurate results are needed the PAH must be administered by constant infusion. The coefficient of variation will then be about 10 per cent.

Example. Patient with hypertension (Fig. 10).

		Colorimeter Readings for *PAH* (*para*-aminohippuric acid).				
		Duplicates		Mean		Derived from graph
Plasma filtrates	B_1	23.4	22.2	22.8	Bm	21.9
	B_2	19.5	20.6	20		
	B_3	19	20.2	19.6	Bn	19.8
Urines diluted 1/1000	U_1	45	44.6	44.8		
	U_2	39.5	38.5	39		

U_1 vol. 127 ml.
Time of 1st period clearance 32 minutes.
U_2 vol. 120 ml.
Time of 2nd period clearance 32 minutes.

$$ERPF_1 = \frac{127}{32} \times \frac{44.8 \times 1000}{21.9 \times 20} = 405 \text{ ml. per min.}$$

$$ERPF_2 = \frac{120}{32} \times \frac{39 \times 1000}{19.8 \times 20} = 368 \text{ ml. per min.}$$

Mean 386 ml. per min.
Volume of packed cells (hæmatocrit) = 45 per cent.
Therefore whole blood flow = 702 ml. per min.

THIOSULPHATE CLEARANCE

Estimation of Thiosulphate. Thiosulphate is titrated directly with dilute standard iodine using starch indicator.

Plasmas B_0, B_1, B_2 and B_3 (2 ml. of each) are diluted with 14 ml. of water, and the proteins precipitated with 2 ml. of 10 per cent sodium tungstate and 2 ml. 2/3 N-sulphuric acid (p. 14). The mixtures are shaken and filtered or centrifuged after 5 minutes.

Urines U_0, U_1 and U_2 are diluted 1 in 50 and 1 in 100 with distilled water.

To 1 ml. of 2 N-hydrochloric acid are added 5 ml. of plasma filtrate and 2 drops of starch solution. Titration is made with N/1000 iodine until a blue colour develops, using a 5 ml. microburette.

3 ml. of dilute urine are titrated similarly. The titrations of the blood blank filtrate (B_0) and the diluted urine blank (U_0) are subtracted from the blood and urine titrations B_1, B_2 and B_3, and U_1, U_2 and U_3.

Calculation. It is unnecessary to calculate the absolute concentrations of thiosulphate as the ratio of the titrations is

sufficient. The blood titration figures are plotted on graph paper as in the case of PAH, and B_m and B_n titrations obtained by intrapolation (see Fig. 10). The calculation is as follows :—

$$\text{Glomerular Filtration Rate* (GFR)} = \frac{\text{V (vol. of urine+washings in ml.)}}{\text{T (time of urine collection in min.)}}$$

$$\times \frac{\text{U (titration)} \times \text{dilution of urine}}{\text{Amount diluted urine titrated (ml.)}}$$

$$\times \frac{5}{\text{B}_{(\text{m or n})}\text{ titration} \times 10\text{ (plasma dilution)}}$$

* in ml. of plasma filtered per min.

GFR determined in this way is subject to an error resulting from falling blood concentrations of thiosulphate. The coefficient of variation using this technique is about 23 per cent, but can be improved to about 12 per cent by administering the thiosulphate as a constant infusion. As in the case of PAH this error becomes progressively less with increasing degrees of renal damage.

Example.

IODINE TITRATIONS OF THIOSULPHATE (ml.)

		Duplicates		Mean	Subtract blanks		Derived from graph
5 ml. plasma filtrate	B_0	0.10	0.10	0.10			
	B_1	3.97	4.05	4.01	3.91	B_m	3.45
	B_2	2.57	2.55	2.56	2.46		
	B_3	2.02	2.02	2.02	1.92	B_n	2.25
3 ml. urine diluted 1/100	U_0	0.16	0.07	0.115			
	U_1	6.19	6.27	6.23	6.115		
	U_2	4.56	4.54	4.55	4.435		

$$GFR_1 = \frac{127}{32} \times \frac{6.115 \times 100}{3} \times \frac{5}{3.45 \times 10} = 117 \text{ ml. per min.}$$

$$GFR_2 = \frac{120}{32} \times \frac{4.435 \times 100}{3} \times \frac{5}{2.25 \times 10} = 123 \text{ ml. per min.}$$

Mean 120 ml. per min.

$$\text{Filtration Fraction FF} = \frac{\text{GFR}}{\text{ERPF}} \times 100 = 31 \text{ per cent}$$

SOLUTIONS

Sodium Thiosulphate. 10 g. of anhydrous $Na_2S_2O_3$ are dissolved in approximately 25 ml. pyrogen-free distilled water and sterilized by boiling. This should be injected intravenously very slowly.

p-*Aminohippuric Acid.* 1 g. is neutralized to pH 7 with sodium hydroxide and made up to 5 ml. with pyrogen-free distilled water, sterilized by boiling, and immediately before injection mixed with approximately 2 ml. of percaine (not novocaine or procaine).

Sulphonamide Reagents. See p. 29.

Thiosulphate Reagents. 10 per cent sodium tungstate and 2/3 N-sulphuric acid (see p. 14); 2 N-hydrochloric acid (p. 190); starch solution (p. 189); N/1000 iodine: the N/10 iodine (p. 191) should be suitably diluted with distilled water immediately before use.

Chapter XI

SPECTROSCOPIC PROCEDURES

In many cases it is useful to examine solutions spectroscopically to identify the pigments present. Many pigments give characteristic dark absorption lines in the visible part of the spectrum, and these lines may be detected and their position in the spectrum approximately determined by direct visual examination with some sort of simple spectroscope.

DIRECT-VISION SPECTROSCOPE

A form of this instrument is illustrated.

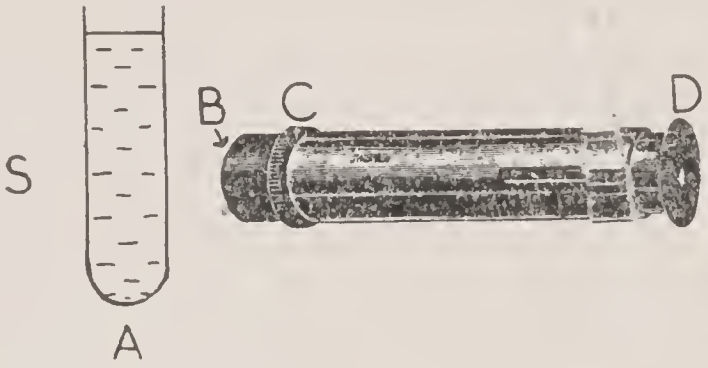


Fig. 11

Light from a source S travels through the solution A and reaches the eye after spectroscopic resolution by means of a prism and lens system in the instrument. The size of the slit B may be adjusted by means of the screw C and the spectrum is focused by moving the eyepiece, D. For the examination of solutions, the eyepiece is placed close to the eye, and the apparatus pointed at a bright light, preferably daylight. The spectrum is sharply focused, and the test-tube or glass cell containing the solution is placed so that it touches the slit B. The spectrum should now be clearly and sharply visible; if not, the slit B, and the eyepiece D, are adjusted

until this condition is attained. The dark bands due to the pigment ought now to be visible.

The following are characteristic :—

Pigment	No. of bands	Position on the spectrum (Wave-length in mμ)
Reduced hæmoglobin	1	565
Oxyhæmoglobin	2	540, 578
Carboxyhæmoglobin	2	535, 572
Methæmoglobin	4	500, 540, 579, 630
Alkaline methæmoglobin	2	541, 580
Sulphæmoglobin	3	540, 578, 618
Hæmochromogen	2	526, 557
Acid hæmatin (in dilute HCl)	5	505, 540, 580, 638, 650
Alkaline hæmatin	1	607
Acid porphyrin	2	554, 600
Alkaline porphyrin	4	504, 539, 576, 622
Stercobilin (urobilin)	1	490

450 mμ	500	550	600	700
Violet blue	blue green	yellow green	yellow orange	r e d

METHODS

Blood

Blood is diluted 1 in 5, or 1 in 10, with water, and cell membranes centrifuged out or allowed to settle. The clear solution is examined in a glass cell or tube. It is important that the greatest possible depth or concentration of solution (consistent with visibility) should be examined, and that a careful search should be made (with varying depths or concentrations of solution) for any bands in the red part of the spectrum (620–630 mμ). The bands due to met- or sulphhæmoglobin, present sometimes in blood from patients treated with drugs (e.g. sulphanilamide and its derivatives), are not always easy to detect. If such bands (at approx. 620–630 mμ) are seen, the solution should be treated with a drop of yellow ammonium sulphide. A band due to methæmoglobin will then disappear; if sulphæmoglobin is present its band

persists. For comparisons, laked blood may be treated with a little potassium ferricyanide solution, which will cause methæmoglobin to be formed. A sample of sulphæmoglobin may be prepared from blood (10 ml. of 1/100 dilution), phenylhydrazine hydrochloride solution (0.1 ml. of a 0.1 per cent solution), and a drop of water saturated with hydrogen sulphide.

Urine

Urine may be directly examined (after filtration or centrifuging) for urobilin, porphyrin, and hæmoglobin pigments.

Fæces

(*a*) **For Stercobilin.** Approximately 1 g. of fæces in a test tube is shaken well with 15 ml. of acid alcohol (1 ml. of conc. hydrochloric acid per 100 ml. of alcoholic solution). After some hours, the supernatant liquid (with dilution, if necessary, with acid alcohol) is examined spectroscopically. The acid alcohol converts stercobilinogen into stercobilin.

(*b*) **For Blood.** About 1 g. of fæces is well shaken in a glass-stoppered cylinder with 10 ml. of water. 10 ml. of glacial acetic acid and 20 ml. ether are now added, and the mixture carefully shaken. The emulsion is allowed to separate (with addition, if necessary, of a little more ether or water). The ether layer is examined for alkaline porphyrin and is then decanted and shaken with 10 ml. of 2 N-hydrochloric acid. Each layer is examined. The ether may contain acid hæmatin (from *blood*) and chlorophyll, and the aqueous part, acid porphyrin. Stercobilin may be present in both layers.

THE QUANTITATIVE ESTIMATION OF CARBON MONOXIDE IN BLOOD

HARTRIDGE REVERSION SPECTROSCOPE

In cases of carbon monoxide poisoning, the blood may appear a bright carmine in colour, due to the presence of carboxyhæmoglobin. The spectrum of carboxyhæmoglobin is similar to that of oxyhæmoglobin, but the bands (α and β) in the green are shifted slightly towards the violet end of the

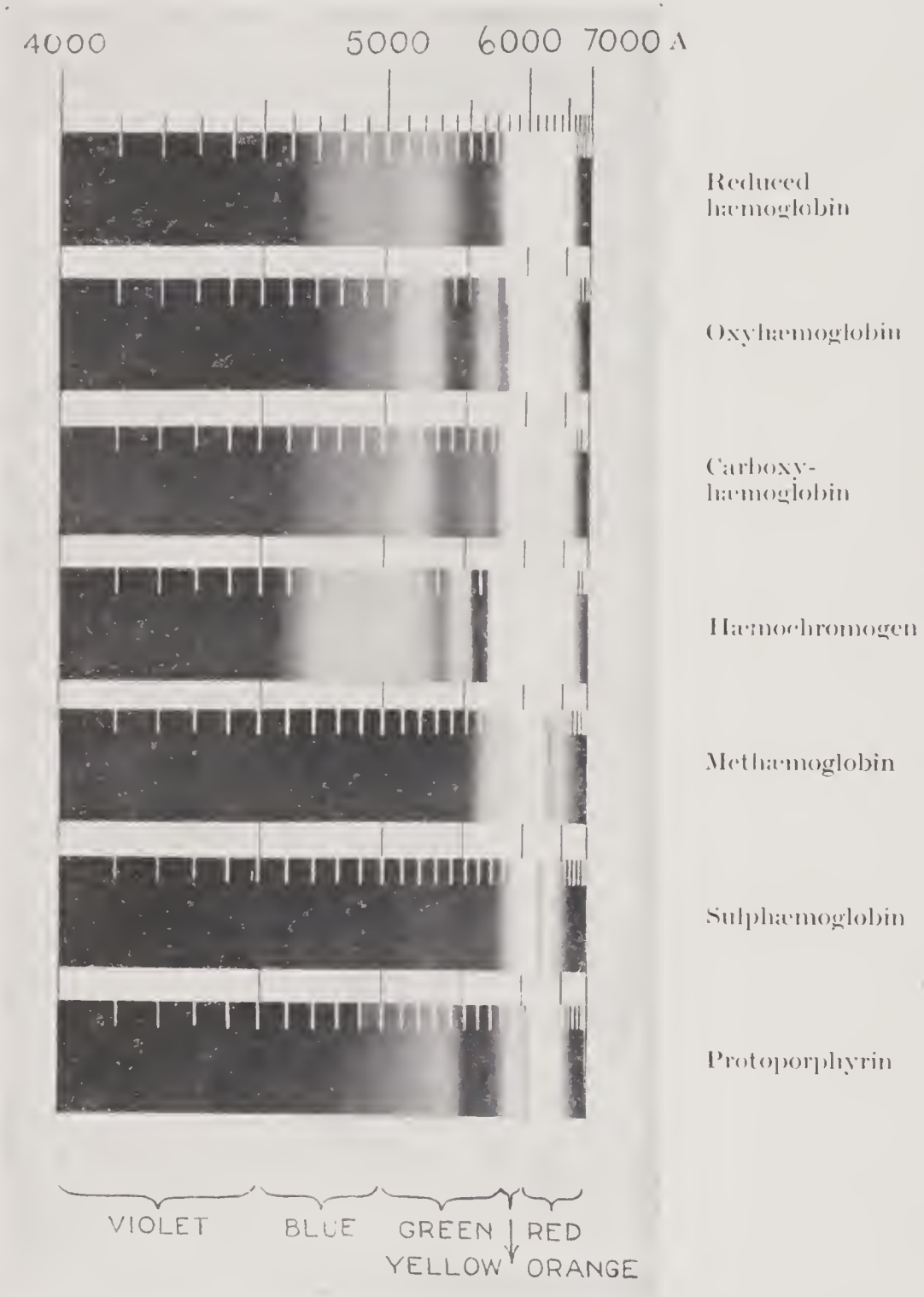


FIG. 12. Absorption spectra of hæmoglobin and its derivatives. (From Thorpe's *Biochemistry*.) The wave-length scale A is in Ångstrom units. 10 A = 1 mμ. (compare table p. 179).

[*To face page* 180

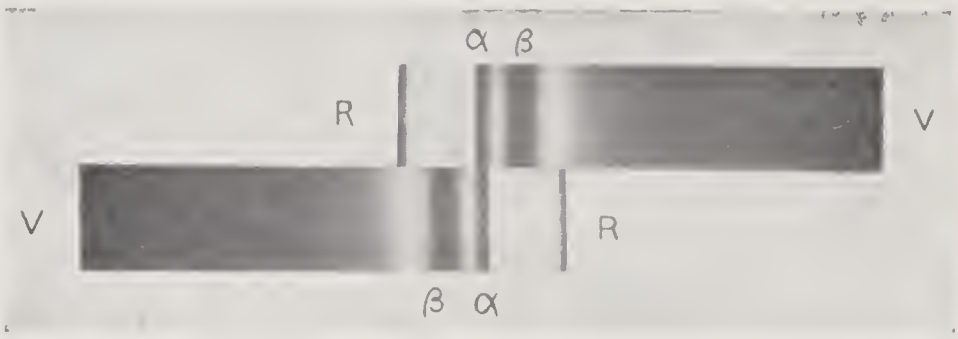


FIG. 13. Spectrum of oxyhæmoglobin in Hartridge reversion spectroscope; adjusted to make *a* lines correspond. (From Thorpe's *Biochemistry*.)

FIG. 14. Spectrum of carboxyhæmoglobin in Hartridge reversion spectroscope; showing shift of *a* bands from their position in oxyhæmoglobin. (From Thorpe's *Biochemistry*.)

spectrum. This shift, though difficult to detect with a simple spectroscope, may be quantitatively measured by means of the Hartridge Reversion Spectroscope. In this instrument light from a source passes through a solution in such a way that two spectra are formed, one immediately above the other.

The spectra are also reversed with respect to one another, and the position of one of them can be altered longitudinally by moving a micrometer screw. If a glass cell filled with a solution of oxyhæmoglobin is placed in the path of the light the corresponding lines (α) in the spectra may be made to coincide (Fig. 13). If, now, the cell is replaced by one containing some carboxyhæmoglobin, the bands will appear shifted with respect to one another (Fig. 14). By adjusting the micrometer screw until coincidence is again obtained, the extent of the shift is measured and can be related to the percentage of carboxyhæmoglobin by reference to a graph, obtained as follows :—

Blood is diluted with ammonia solution (4 ml. of conc. ammonia per litre in water). The dilution of blood (usually 1 in 20) must be such that, with the cell used, the distance between the α and β hæmoglobin bands is approximately equal to the width of one of them. Portions of the solution are saturated with oxygen to give 100 per cent oxyhæmoglobin, and with coal gas to give 100 per cent carboxyhæmoglobin solutions. The two cells A and B are then placed face to face so that the light traverses them both. Cell A is first filled with 100 per cent oxyhæmoglobin and B with ammonia solution. A reading is taken at coincidence of the α hæmoglobin bands. Cell A is now emptied and refilled with 100 per cent carboxyhæmoglobin solution and another reading taken, at coincidence. For 50 per cent saturation of carboxyhæmoglobin, cell A is filled with a mixture of equal volumes of dilute ammonia and 100 per cent carboxyhæmoglobin solution and cell B with a similar mixture of ammonia and 100 per cent oxyhæmoglobin solution. For 25 per cent saturation of

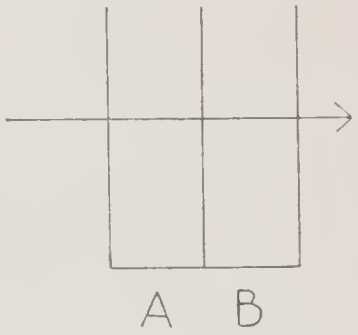


FIG. 15.

carboxyhæmoglobin, cell A contains a mixture of 25 volumes of 100 per cent carboxyhæmoglobin and 75 volumes of dilute ammonia, while cell B is filled with a mixture of 75 volumes of oxyhæmoglobin and 25 volumes of dilute ammonia. Similarly, points for other concentrations of carboxyhæmoglobin are obtained. In each case, coincidence of the α line is obtained and the micrometer reading noted. The readings for the various concentrations are subtracted from that for 100 per cent oxyhæmoglobin, and the results plotted as shown.

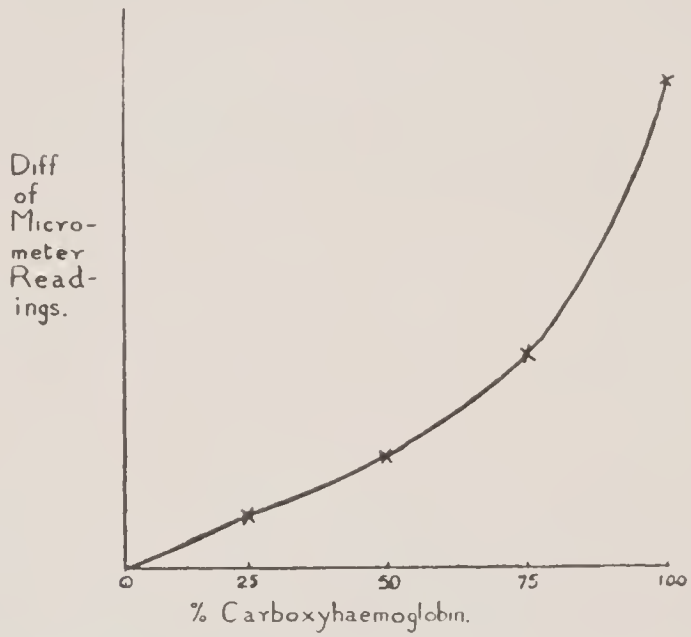


FIG. 16.

For the estimation, a 100 per cent oxyhæmoglobin solution of the above dilution is placed in a cell, and coincidence obtained. The cell is now replaced by another containing the suspected blood, similarly diluted, and the amount of shift (x) necessary to obtain coincidence is measured. It is advisable to check the instruments, also, with a 100 per cent carboxyhæmoglobin solution. The results obtained for this and for 100 per cent oxyhæmoglobin should agree with the standard graph. When this has been done, the amount of shift (x) given by the suspected blood can be related to percentage saturation with carboxyhæmoglobin by simple reference to the curve.

Chapter XII

HYDROGEN ION CONCENTRATION

In pure distilled water the concentrations of hydrogen ions and of hydroxyl ions are equal. The water is 'neutral.' In an acid solution there is a preponderance of hydrogen ions over the concentration of hydroxyl ions. In an alkaline solution the concentration of hydroxyl ions is greater than that of hydrogen ions.

The product of the concentrations of hydrogen ions and of hydroxyl ions is the same (i.e. is a constant) in all aqueous solutions, be they acid, neutral or alkaline.

$$[H^+] \times [OH^-] = K_w$$

Pure distilled water has a concentration of one ten millionth gram of hydrogen ions per litre, i.e. $1/10^7$ or more briefly a concentration of 10^{-7} gram ions per litre. The concentration of hydroxyl ions in distilled water is likewise 10^{-7} g. ions per litre. The product of the concentration of hydrogen ion and hydroxyl ion is $10^{-7} \times 10^{-7} = 10^{-14}$, the value of the constant K_w.

Hundredth-normal hydrochloric acid (0.01 N-HCl) has a concentration of hydrogen ion of about 0.01 g. ions per litre, i.e. of 10^{-2}. The hydroxyl ion concentration will, therefore, be 10^{-12} (and the value of the product remains 10^{-14}). Likewise for hundredth-normal sodium hydroxide (0.01 N-NaOH) the concentration of hydroxyl ions is 10^{-2} and of hydrogen ions 10^{-12}. And the constant K_w is 10^{-14}.

The symbol pH is used to denote the negative logarithm (to the base 10) of the hydrogen ion concentration, e.g. pH 7 for the distilled water of hydrogen ion concentration 10^{-7}, pH 2 for the 0.01 N-hydrochloric acid of hydrogen ion concentration 10^{-2}, and pH 12 for the 0.01 N-sodium hydroxide whose concentration of hydrogen ions is 10^{-12}. It forms

a convenient means for designating the hydrogen ion concentration of any solution. In most biological fluids the hydrogen ion concentrations vary only within narrow limits, and do not depart very markedly from the neutral point of pH 7.

The colorimetric determination of pH is dependent on the use of indicators, organic compounds which change colour with change in hydrogen ion concentration. The changes in colour of indicators take place over definite and fairly narrow ranges of hydrogen ion concentration, within which intermediate shades of colour corresponding to different values of pH can be recognized. The following is a list of the indicators most commonly used in biological work :—

Indicator	pH *range and colour change*
Thymol blue	red 1.2 to 2.8 yellow.
Töpfer's indicator (dimethyl yellow)	red 2.8 to 4.5 yellow.
Methyl orange	red 2.9 to 4.6 yellow.
Congo red	blue 3.0 to 5.0 red.
Bromphenol blue	yellow 3.0 to 4.6 blue.
Methyl red	red 4.2 to 6.3 yellow.
Bromthymol blue	yellow 6.0 to 7.6 blue.
Phenol red	yellow 6.8 to 8.4 red.
Thymol blue	yellow 8.0 to 9.6 blue.
Phenolphthalein	colourless 8.2 to 10.0 red.

METHOD

The pH of an unknown liquid may be determined by adding 1 ml. of a dilute solution of the indicator to 10 ml. of the liquid in a test tube. The tube is stoppered, the contents mixed, and the colour compared with those in a standard set of buffered solutions of known pH containing the same amount of indicator. In the case of coloured solutions (e.g. urine) it is convenient to compensate for the colour by placing a tube containing the properly diluted coloured solution behind the standard indicator tube, and a tube of water behind that containing coloured solution plus indicator. A special comparator apparatus is best used for this purpose. An approximate pH value for the liquid can be obtained by noting the colour produced with one or more indicators and

comparing with the table. Thus, if urine is yellow to bromthymol blue and is yellow or orange yellow to methyl red, its pH is probably in the neighbourhood of 6.0. For fuller discussion and instructions see: Britton (1942), and Clark (1928). A convenient comparator, complete with tubes of standard buffers with indicators, for the colorimetric determination of pH is obtainable from Messrs. British Drug Houses and Messrs. Hopkin & Williams. The B.D.H. Capillator set is a useful instrument for pH measurements of very small, as well as large, quantities of liquid. A convenient pH comparator set containing permanent artificial standards of coloured glass is made by the Lovibond Tintometer and is sold by British Drug Houses.

SOLUTIONS

The indicators mentioned above may conveniently be purchased in ready-made solutions. If it is desired to prepare them from the solid dyes, then 0.1 g. of thymol blue, bromphenol blue, bromthymol blue or phenol red should be dissolved in 20 ml. of warm alcohol and diluted to 100 ml. with water. Preparation of Töpfer's indicator is given on p. 155. and of methyl orange, methyl red and phenolphthalein on p. 191. British Drug Houses' universal indicator is a useful reagent for the quick determination of the approximate pH of liquids.

INDICATOR PAPERS

Messrs. Johnson and Sons, Hendon, N.W.4, and British Drug Houses supply indicator papers—strips of filter paper impregnated with the indicator substances together with colour charts showing the colours given by different pH's. With these papers the pH of any solution may be determined very quickly, and accurately enough for many purposes.

Chapter XIII

VOLUMETRIC SOLUTIONS*

A 'NORMAL' solution of any substance contains the equivalent of 1 gram of hydrogen (capable of reaction) in 1 litre of solution. Acids furnish the reactable hydrogen directly; they are said to have 'replaceable' hydrogen. But in many cases the relation of the substance to hydrogen is only obtained through an intermediate series of reactions.

NORMAL SULPHURIC ACID

Sulphuric acid ($H_2SO_4 = 98$) clearly contains 2 g. of hydrogen per gram mole, and hence $\frac{1}{2}$ gram mole ($= 98/2 = 49$ g.) of sulphuric acid is present in one litre of N-sulphuric acid. In practice, 27 ml. of concentrated acid (sp. gr. 1.84; hence 27 ml. approx. $\equiv$ 49 g.) are measured into about 800 ml. of cold water in a litre volumetric flask. When the solution is cold it is made to the mark and well mixed. This approximately N-acid is then standardized against pure anhydrous sodium carbonate by the reaction—

$$Na_2CO_3 + H_2SO_4 \longrightarrow Na_2SO_4 + CO_2 + H_2O$$

From this equation, 106 g. of sodium carbonate is equivalent to 2 l. of N-sulphuric acid, or 1.06 g. sodium carbonate $\equiv$ 20 ml. of N-sulphuric acid. About 1 g. of anhydrous sodium carbonate is therefore accurately weighed out on to a watch glass, and washed into a 100 ml. flask or beaker. To this solution, a few drops of methyl orange indicator are added, and the approximately N-sulphuric acid is run in from a burette until the indicator changes from yellow to orange (it is red in acid solution, yellow in alkaline). The experiment is repeated.

* The subject is treated only very briefly in this chapter. Recourse may be had to such standard text books of analysis as Treadwell and Hall (1935-42) and Scott (1939).

From the above equation, the number of ml. of the approximately N-sulphuric acid which would be equivalent to 1.06 g. of sodium carbonate is calculated. This should be less than 20 ml. Suppose, for example, it is 18.45 ml.; then every 18.45 ml. of the acid is equivalent to 20 ml. of N-acid. That is, 18.45 ml. of acid must be diluted with water to 20 ml., to give N-acid. An appropriate dilution of part or all of the remaining approximately N-acid is now made.

The mixed solution should be exactly N, and may be again tested against sodium carbonate to confirm this.

NORMAL SODIUM HYDROXIDE

Sodium hydroxide (NaOH=40) contains in 40 g. of substance an amount of sodium (23 g.) which is equivalent to 1 g. of hydrogen. This 'equivalence' of sodium to hydrogen is best understood by reference to the equation—

$$HCl+NaOH=NaCl+H_2O.$$

Approximately 41 g. of the solid are weighed out as quickly as possible, and put into 50 ml. of water in a litre flask. When cold, the solution is mixed and made to volume. This approximately N-solution is standardized against pure oxalic acid.

$$2NaOH+\begin{vmatrix} COOH \\ COOH \end{vmatrix} \longrightarrow \begin{vmatrix} COONa \\ COONa \end{vmatrix} +2H_2O$$

90

Hence, 2 litres of N-sodium hydroxide are equivalent to 90 g. of anhydrous oxalic acid, or 90+36=126 g. of oxalic acid crystals, $\begin{vmatrix} COOH \\ COOH \end{vmatrix} .2H_2O$

Thus,

1.26 g. of oxalic acid crystals

≡20 ml. of N-sodium hydroxide.

Therefore about 1.2 g. of oxalic acid are accurately weighed out; washed into a flask, and the solution titrated with the

approximately N-sodium hydroxide with phenolphthalein (pink in alkaline, colourless in acid solution) as indicator. The amount of the alkali equivalent to 1.26 g. of oxalic acid is calculated. This amount should be less than 20 ml. The dilution to exactly N is then accomplished, as in the case of sulphuric acid.

NORMAL AMMONIA SOLUTION

Concentrated (sp. gr. 0.88) ammonia solution is approximately 17N. 58 ml. of this are therefore diluted with water to 1 litre, and the solution standardized against the N-sulphuric acid, with methyl red as indicator.

TENTH NORMAL POTASSIUM PERMANGANATE

Potassium permanganate ($KMnO_4$) reacts in acid solution with oxalic acid as follows :—

$$\underset{158}{2KMnO_4} + 5\underset{\substack{\text{COOH}\\ \text{COOH}\\ 90\\ (+2H_2O\\ =126)}}{\left|\begin{matrix}\text{COOH}\\ \text{COOH}\end{matrix}\right.} + 3H_2SO_4 \longrightarrow K_2SO_4 + 2MnSO_4 + 10CO_2 + 8H_2O$$

1 gram mole of potassium permanganate is equivalent to 5 atoms of hydrogen. Hence, 0.1 N-permanganate contains $\frac{158}{50} = 3.16$ g. per litre of solution. 3.16 g. of pure potassium permanganate are therefore weighed out, dissolved in water, and the volume made to 1 litre.

From the above equation, 5×126 g. of oxalic acid crystals $\equiv$10 l. of N, $\equiv$100 l. of 0.1 N-permanganate. That is, 0.126 g. of oxalic acid crystals $\equiv$20 ml. of 0.1 N-permanganate. The above 0.1 N-solution may therefore be checked by titrating with it an exactly weighed amount (about 0.126 g.) of pure oxalic acid crystals, dissolved in approximately N-sulphuric acid. The acid solution is held in a beaker of hot water (about 80° C.) during titration, which is continued until the first persistence of pink permanganate colour.

TENTH NORMAL SODIUM THIOSULPHATE

Sodium thiosulphate ($Na_2S_2O_3$) reacts with iodine as follows:—

$$2Na_2S_2O_3 + I_2 \longrightarrow 2NaI + Na_2S_4O_6$$

Hence, 1 gram mole of thiosulphate≡1 gram atom of iodine and (since iodine reacts with hydrogen to give hydriodic acid, HI) 1 gram mole of thiosulphate is contained in 1 litre of N-solution. Approximately 0.1 N-sodium thiosulphate is made by dissolving 25 g. of the crystals ($Na_2S_2O_3.5H_2O \equiv 248.2$) in water and making to 1 litre.* This solution is then standardized against a known amount of iodine, obtained from potassium biiodate ($KIO_3.HIO_3$) solution by the reaction—

$$\underset{389.97}{KIO_3.HIO_3} + 10KI + 11HCl \longrightarrow 11KCl + 6I_2 + 6H_2O$$

0.1 N-potassium biiodate is therefore made by dissolving $\frac{389.97}{10 \times 12} = 3.250$ g. of pure potassium biiodate in water in a litre flask, and making to volume. The titration is carried out as follows :—

A flask containing a solution of approximately 1 g. of potassium iodide in a little water is treated with about 10 ml. of approximately 5 N-hydrochloric acid (made by diluting the concentrated acid with an equal volume of water). 25 ml. of the 0.1 N-potassium biiodate solution are then run in from a pipette. The iodine set free is titrated with the approximately 0.1 N-sodium thiosulphate, until a light yellow colour is obtained. Two drops of starch indicator are added, and the titration continued until the solution is colourless. The titration should be less than 25 ml. Dilution of the thiosulphate to exactly 0.1 N is carried out as in the previous cases.

Starch Indicator. 100 ml. of water are heated in a beaker to boiling. A paste of approximately 1 g. of starch in a little

* 1 ml. of 20 per cent sodium carbonate and 10 ml. of amyl alcohol per 1 may be included in the solution as a preservative.

TABLE 9

Table of Normalities

Sp. Gr. at 15°C.	Normality	ml. conc. per litre to give normality
	SULPHURIC ACID	
1.016	0.5	13.9
1.032	1.0	27.8
1.063	2.0	55.5
1.153	5.0	138.9
1.290	10.0	277.7
1.840	35.9	—
	HYDROCHLORIC ACID	
1.008	0.5	50
1.017	1.0	100
1.034	2.0	200
1.083	5.0	500
1.16	10.0	—
	NITRIC ACID	
1.017	0.5	31.7
1.033	1.0	63.4
1.067	2.0	126.7
1.165	5.0	316.8
1.305	10.0	633.6
1.420	15.75	—
	ACETIC ACID	
1.0037	0.5	30
1.0083	1.0	60
1.0171	2.0	120
1.0412	5.0	300
1.0685	10.0	600
1.0553	16.16	—
	AMMONIUM HYDROXIDE	
0.9963	0.5	28.6
0.9925	1.0	57
0.9854	2.0	114
0.9620	5.0	286
0.9313	10.0	571
0.9000	15.0	872
0.882	17.2	—

cold water is poured into the beaker. A few crystals of phenol red are added, and boiling is continued for a few minutes. The solution is cooled and preserved with a little chloroform.

TENTH NORMAL IODINE SOLUTION

Approximately 13.5 g. of pure sublimed iodine are dissolved in a solution of 24 g. potassium iodide in about 100 ml. of water in a litre volumetric flask. The solution is diluted to the mark, mixed, and standardized against 0.1 N-sodium thiosulphate.

INDICATOR SOLUTIONS

Methyl Orange. 0.1 g. of methyl orange is dissolved in 100 ml. of water.

Methyl Red. 0.2 g. of methyl red in 100 ml. of alcohol.

Phenolphthalein. 0.5 g. of phenolphthalein is dissolved in 50 ml. of alcohol and 50 ml. of water are added.

Chapter XIV

COLORIMETRIC AND PHOTOMETRIC MEASUREMENTS

COLORIMETRIC ANALYSIS

Many substances of biological and medical interest are coloured. Others form coloured derivatives ; and still others can be made to enter into chemical reactions which yield coloured substances. The measurement of the concentration of coloured substances, usually in solution, forms the basis of colorimetric analysis.

The simplest way of measuring the concentration of a coloured substance is to compare the colour of a measured amount of its solution in a test tube with the colours of known standard solutions of varying concentrations of the same substance in similar test tubes. The concentration of the substance in the standard test tube which matches that of the unknown is taken to represent the concentration of the solution under test. Since their colours are similar their concentrations must be similar.

Nessler Tubes

The best known example of this simplest form of colorimetric analysis is that in which Nessler tubes are used for determining the amount of ammonia in water. Several different measured amounts of a standard ammonia solution are placed in the Nessler test tubes. All are then diluted to the same volume with distilled water. In another Nessler tube is now placed a like volume of the water under examination ; and to all the tubes are added the same amount of Nessler solution. The yellow colour produced is viewed either by looking horizontally through the test tubes or vertically by looking down through them ; and the standard tube whose colour most nearly matches that of the water being examined is sought.

The concentration of any coloured substance, or of any substance which is capable of yielding a coloured derivative or other coloured substance in a chemical reaction, can easily be determined in this manner in any set of test tubes provided they are of similar bore. But an exact match with the unknown coloured solution may not be obtained ; its colour may appear to lie between those of two of the standards, and its exact concentration must therefore be guessed at. As a consequence this form of colorimetric analysis is not capable of yielding results of the highest accuracy. Moreover, the preparation of multiple standard solutions is bothersome. Simpler and more precise colorimetric comparisons may be made with a single standard coloured solution.

Hehner Tubes

A simple refinement of the Nessler-tube technique consists in the following. Two 20 ml. graduated cylinders are selected which are made of similar glass, and have good clear bottoms. Their internal diameters must be the same, and, consequently, the height of the different points of graduation must be similar, i.e. each ml. mark of one tube is the same distance from the bottom as it is in the other. This means that, when the two cylinders contain similar volumes of coloured solution, columns of liquid of the same depth will be looked at when viewed vertically from the top downwards ; and if the similar volumes are of the same coloured solution then their colours will appear the same. If, now, a dilution of the coloured solution be made so that its concentration is half that of the original, and similar volumes are placed in the two cylinders, then the original solution will obviously appear to be darker in colour than the diluted one. But if 10 ml. of the original solution is placed in one cylinder and 20 ml. of the diluted half-strength solution in the other, then their colours will appear similar when viewed vertically, since one is looking through twice the length of 50 per cent solution as one is of the 100 per cent solution.

By sealing a glass stopcock in the side of one of the cylinders near its base a simple colorimeter known as the Hehner tube

may be made (Fig. 16 Gallenkamp Mo. 15217). The cylinder with the stopcock is filled to its 20 ml. mark with the 100 per cent coloured solution, and the other cylinder similarly with 50 per cent coloured solution. The two cylinders are mounted side by side over a white background placed at an angle to the light from a window, so that it is reflected upwards through the coloured solution. This is most easily done by placing them on a small sheet of glass on top of a wire basket in which has been placed a piece of white cardboard set at an angle of about 45° to vertical. The 100 per cent coloured solution is now allowed to run out through the stopcock into a small beaker until the colour in its cylinder appears of the same depth as that in the cylinder containing the 20 ml. of 50 per cent solution. At this point it will be found that the volume of 100 per cent solution remaining in its cylinder is 10 ml. or nearly so. 10 ml. of 100 per cent solution appear to have the same depth of colour as 20 ml. of 50 per cent solution. In fact, one is looking at the same amount of coloured substance in 10 ml. of 100 per cent solution as is contained in 20 ml. of 50 per cent solution, and their colours, therefore, appear similar. This simplest and cheapest of all colorimeters can be used for any colorimetric estimation. The weaker of any two coloured solutions is placed in the cylinder without the stopcock and the stronger in that with the stopcock. The latter is run out until the depth of colours in the two cylinders appear to be the same. The volume of the stronger solution is then noted. The solution which has been run out into the beaker is restored to the cylinder and the operation repeated. In this way several readings may be taken and averaged. The concentrations of the two coloured solutions are in inverse ratio to the volumes in the two cylinders at the point where their colours appear to match. Thus we have the relation : concentration of test × its volume = concentration of standard × its volume. For the purpose of

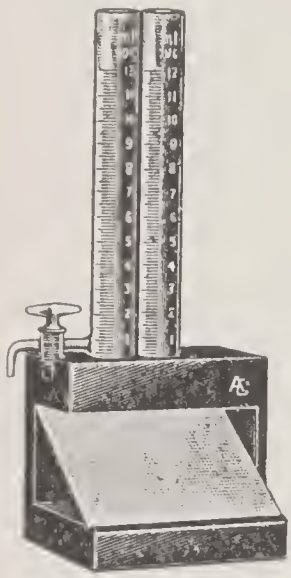


Fig. 17.

calculating the concentration of an unknown solution it becomes

$$\text{Concentration of test} = \frac{\text{Volume of standard}}{\text{Volume of test}} \times \text{Concentration of standard}$$

A more precise statement of this relationship is known as Beer's Law. This states that the amount of light absorbed by a coloured solution is proportional to the total number of light-absorbing molecules in the light path, and is independent of their concentration.

Duboscq Colorimeter. This precise instrument makes very much more accurate the colorimetric comparisons described above. The construction is illustrated in Fig. 18. It consists of a pair of adjustable glass cups and plungers, for adjusting the depth of coloured solution through which two beams of light from a single source are made to pass. The light coming through the glass plungers traverses the two sides of a prism into a system of lenses, which bring the two light beams into an eyepiece. There they appear as a circular patch of coloured light, which is divided into two semicircles in close juxta-position and separated by a very narrow dividing line (which is the image of the edge of the prism).

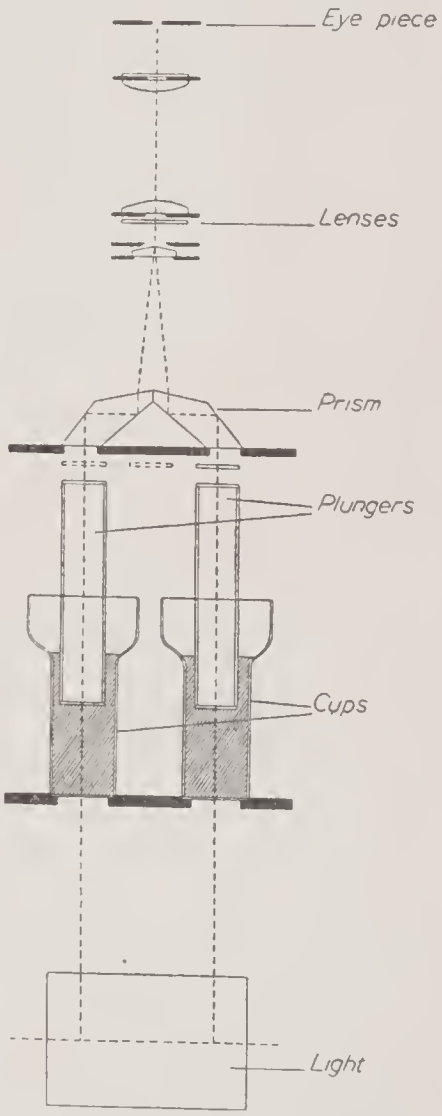


FIG. 18. Diagrammatic representation of the Duboscq colorimeter. (Bausch and Lomb.)

When the same coloured solution is placed in both cups, and their positions are adjusted by means of the rack-and-pinion knobs to equalize the distances between the bottoms of

the cups and the bottoms of the plungers, then similar columns of coloured solution will have been placed in the paths of both beams of light, and the two halves of the circle of coloured light will appear identical. But if the 50 per cent coloured solution be placed in one cup, and its depth be adjusted to 20 mm., then it will be found that, with the 100 per cent solution in the other cup, one has to decrease its depth to 10 mm. in order to achieve equality of colour brightness in the two halves of the field as viewed through the eyepiece. In this way, with any coloured solution of unknown concentration, the depth (in mm.) may be found which is necessary to match the colour of a known standard solution set at a defined depth of, say, 20 mm. If the unknown solution is only half as concentrated it will match at 40 mm. ; if it is twice as concentrated it will match at 10 mm. ; if four times, then at 5 ; and so on. And the concentration of the unknown solution may be derived from the equation

$$\text{Concentration of test} = \frac{\text{Reading of standard}}{\text{Reading of test}} \times \text{Concentration of standard}$$

In operating a Duboscq colorimeter a few points of technique are worth mentioning. All glass surfaces must be scrupulously clean. The cups and plungers must be dry (i.e. free of any previously contained solution), or they must be rinsed with the solution about to be investigated, care being taken to wipe off any drops of liquid from the lip and outside with filter paper. The light source, or the colorimeter relative to the light source if the latter be daylight, must be so adjusted that exactly equal quantities of light enter the instrument at both sides through the cups, plungers, etc. This is most easily accomplished by placing a standard coloured solution in both cups, setting the racks on both sides so that the depths are equal, e.g. at 20 mm. and then adjusting the light so that both sides of the coloured field appear equal. Bubbles of air on the bottoms of the plungers should be looked for when the cups are adjusted to bring the plungers into the liquid, and may be got rid of either by tilting the colorimeter backward or by racking the cup up carefully until its bottom just

touches the plunger. The zero reading should be checked to confirm that the plunger touches the bottom of the cup (i.e. there is zero depth of coloured solution) when the scale reads O. It is usual to set the standard at a defined depth on the left side of the instrument, and to adjust the depth of the unknown to the point of colour match on the right side. When several unknowns are read against a single standard the right-hand cup should be rinsed with each succeeding solution, before the cup is filled and the reading made. Cups and plungers should be rinsed with tap and distilled water at the conclusion of each set of readings, the plungers wiped with a clean towel or paper, and the cups left inverted on filter paper.

PHOTOMETRIC MEASUREMENT WITH THE ORDINARY COLORIMETER

Photometric measurement of the intensity of colour in a solution is preferable, for analytical purposes, to colorimetric comparison with standard solutions. More accurate measurement of the colour is possible, and interference by extraneous colours—a source of frequent trouble in colorimetry—can be avoided. Photometers are, however, expensive, and most laboratories are already equipped with colorimeters of the Duboscq type. By the use of light filters and neutral grey screens it is possible to make photometric measurements with the ordinary Duboscq colorimeter.* By placing the light filter on the top of the eye-piece, and using daylight or artificial illumination, virtually monochromatic light is obtained. The light source is then adjusted to yield exactly equal illumination on both sides of the field. Neutral grey screens serve as standards of light absorption.† The neutral screen is placed on the left-hand rack of the colorimeter, and the rack screwed up till the screen is against the bottom of the plunger. The

* The spectral filters of Messrs. Ilford Ltd. are appropriate for the purpose. A set of eight gelatin spectral filters, mounted in glass (grade "A" glass, $\frac{3}{4}$ in. diameter, is suitable), can be obtained covering the visible spectrum.

† For example, Ilford neutral grey screens of 0.25, 0.50, and 0.75 densities (D). For most purposes the following set is sufficient : Ilford 41/45 violet, 807 mercury green, 205 narrow-cut tricolour red, and 0.5D grey (A glass, 1 in. diameter).

coloured solution is placed in the right-hand cup and its depth is adjusted until the two fields appear equal. This depth gives the measure of the light absorbed by the solution, which is equal to that absorbed by the neutral grey screen. The absorption will vary for lights of different wave-length as given by the different filters. With the filter showing maximum absorption the reading (millimetres of solution) will be minimum; and the depths of two different solutions of the same coloured substance should be in inverse ratio to the strengths of the solutions. Generally speaking, absorption will be found to be maximum for red solutions in the green or blue-green, and conversely, green solutions will show maximum absorption in the red. Blue and violet solutions are maximally absorbing in the yellow, orange, and red; and yellow and orange solutions in the violet and blue.

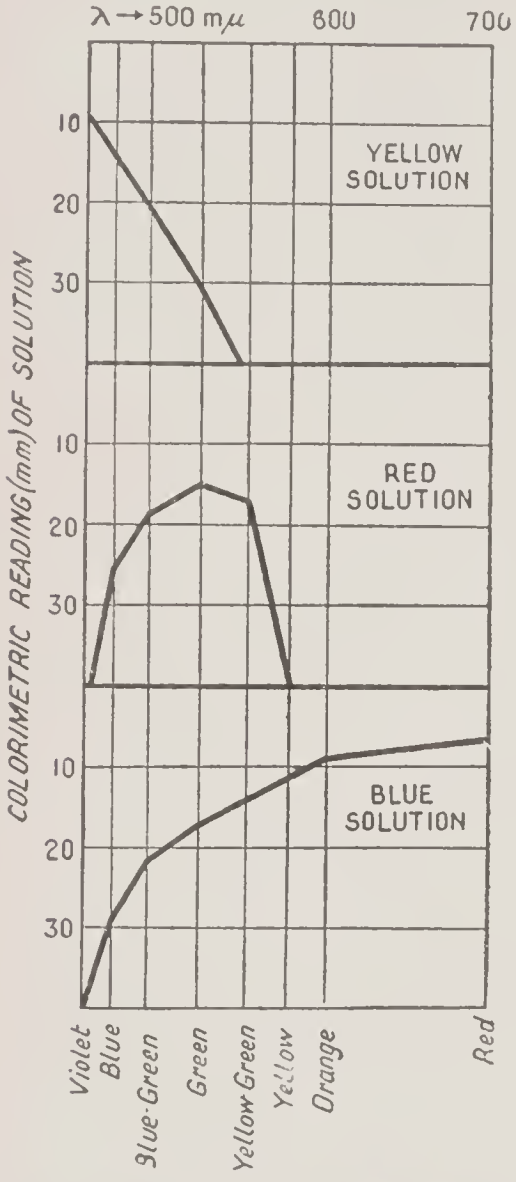


FIG. 19. Absorption curves of standard solutions (neutral grey screens, spectral filters). Yellow solution: nesslerized ammonium chloride solution (strong urea standard); neutral screen, density 0.75. Red solution: bilirubin standard, 0.1 mg. in 25 ml. (Haslewood and King, 1937); neutral screen, density 0.50. Blue solution: uric acid standard; neutral screen, density 0.50.

The grey screen of an appropriate density, together with the light filter showing maximum absorption, may be used as a permanent standard for any colorimetric method. It should be calibrated against the coloured solution of known strength—the 'standard'; and the general equation for calculating the result for an unknown solution—the 'test'—then becomes :—

$$\text{Concentration of test} = \frac{\text{Reading of standard against grey screen}}{\text{Reading of test against grey screen}} \times \text{Concentration of standard}$$

The colour of any solution may also be expressed as its 'extinction coefficient,' (E). The calculation is as follows :—

$$E = \frac{\text{Density of grey screen (e.g. 0.50)}}{\text{Reading of solution against grey screen (in cm.)}}$$

When comparison of an unknown solution with a known standard of the same substance is made, using the light filter showing maximum absorption, the accuracy of matching is increased ; and interference by other contaminating colours is minimized because they are not maximally absorbing for light of the wave-length being used.

NOTE. A fairly powerful light source is required with these filters. If the colorimeter is equipped with an artificial light it may be advantageous to increase the illumination by substitution of a stronger light bulb for that furnished.

GREY-WEDGE PHOTOMETER

King and Delory (1944) described a photometer for field use which was made from a Lovibond comparator whose coloured disks were replaced by nine grey screens of density 0.1–1.0, and whose two fields (grey and coloured) were viewed through a complementary light-filter. In the photometer described by King (1947), King *et al.* (1948), the accuracy and ease of colour measurement are greatly increased by using a continuous grey-wedge in place of the disk of grey screens, and by bringing the two photometric fields together with an eyepiece. The construction is shown in the diagram (Fig. 20). The solution to be examined is placed in the glass cell (2) alongside a similar cell (1) containing the solvent only, which serves to compensate losses due to absorption and reflexion. Both cells are illuminated by daylight or bright diffused lamplight falling on the diffusing screen (3). The two beams of light passing through the cells are brought together into the eyepiece by means of the Hufner prism (4). The field of view consists of two juxtaposed semicircular

patches, each illuminated by the light from one cell only, with no dividing line between them.

Between the control cell (1) and the eyepiece is a continuously variable neutral grey wedge (5) (Ilford, Ltd.), consisting of a circular strip of gelatine, loaded with carbon or silver of uniformly increasing thickness enclosed between two glass discs, and increasing approximately uniformly with the angle of rotation of the disc. A scale is attached to the

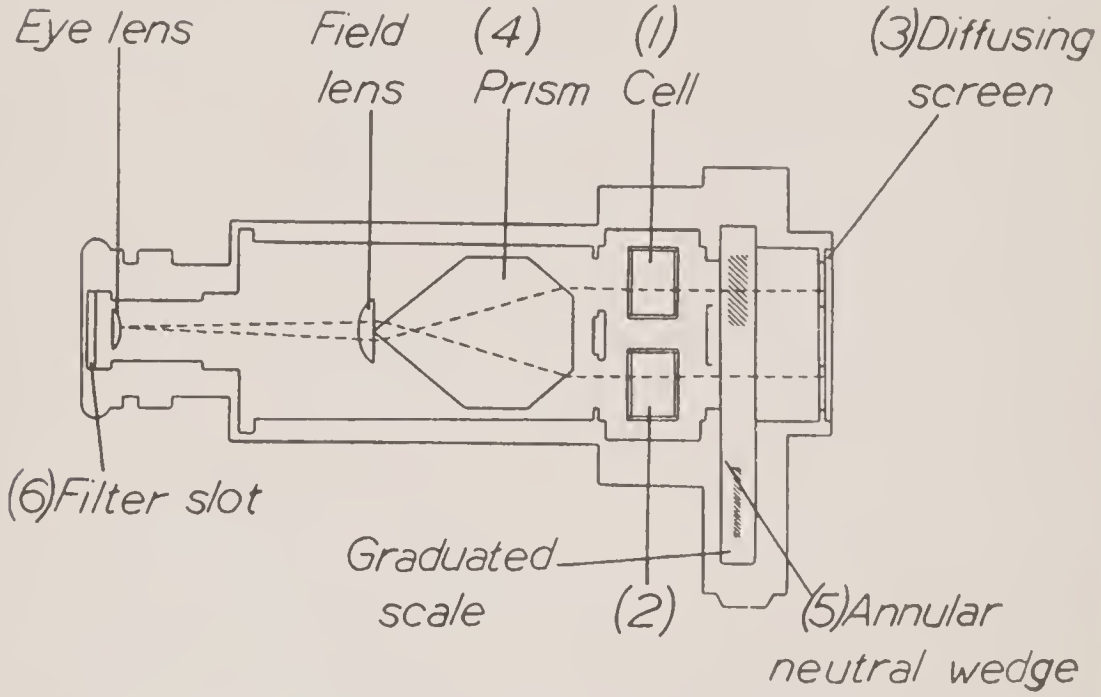


FIG. 20. Optical system of the Grey-wedge Photometer.

periphery of the wedge holder. Interchangeable coloured filters, chosen to suit the particular solution being tested, can be mounted at (6), so that both beams of light must pass through it.

To use the instrument, the cells are placed in position, a suitable filter inserted, the eyepiece sharply focused on the front edge of the Hufner prism, and the whole held up to the light. The neutral wedge is then rotated until the intensities of both halves of the field of view match. The optical density of the solution can be read from the scale.

This photometer may be purchased from Messrs. Keeler Ltd., 39 Wigmore St., W.1. It can be used for a wide range

of chemical estimations, if suitable filters are chosen (e.g. Ilford 205 red for blue solutions, 625 and 807 greens for red and brown solutions, and spectrum violet (41/45) for yellow). It is robust, simple to use and quick in action. Its accuracy is comparable with that of the best Duboscq colorimeters and visual photometers.

DIRECT-READING SINGLE-CELL PHOTOELECTRIC COLORIMETER

Duboscq colorimeters and photometers are now giving place to photoelectric colorimeters, which estimate the intensity of colour in a solution by measuring photoelectrically the proportion of incident light which is absorbed by the coloured solution. Typical instruments are the Evelyn (1936), the Hilger (1936), and the King (1942). When light falls on a photoelectric cell the current generated can be measured by connecting the cell to a galvanometer. The degree of deflexion of the galvonometer needle depends on the amount of current generated, and this depends on the amount of light falling on the photoelectric cell. If a coloured solution is interposed between the source of light and the photoelectric cell some of the light is absorbed, less electricity is generated and a smaller deflexion of the galvanometer takes place. A more strongly coloured solution will absorb more light, still less electricity will be generated, and the deflexion will be still smaller. The degree of deflexion of the galvanometer, therefore, can be used to give a measure of the intensity of colour in the solution, and hence of the concentration of the substance being analysed.

The instrument here described makes use of this simple principle. It has been in use for routine and research in these laboratories since 1940 and has largely displaced the visual comparison colorimeters formerly in use. Some of the advantages of photoelectric colorimetry over visual colorimetry are the much greater speed and ease of colour measurement, and the elimination of eye fatigue and personal error.

CONSTRUCTION

The construction and assembly of the instrument are illustrated in Fig. 21, and are typical of several good commercial models now available (e.g. EEL, Gallenkamp, Hilger, and Unicam). An ordinary torch-type lamp furnishes the source of light (E). The bulb is activated from an accumulator or constant-voltage transformer. A steady, non-fluctuating light is obtained. Immediately in front of the lamp is mounted a block of wood or plastic which has been bored vertically to fit a $\frac{5}{8}$-in. test tube (A) or 1 cm. glass cell. The shutter (B) is used to vary the size of the slot through

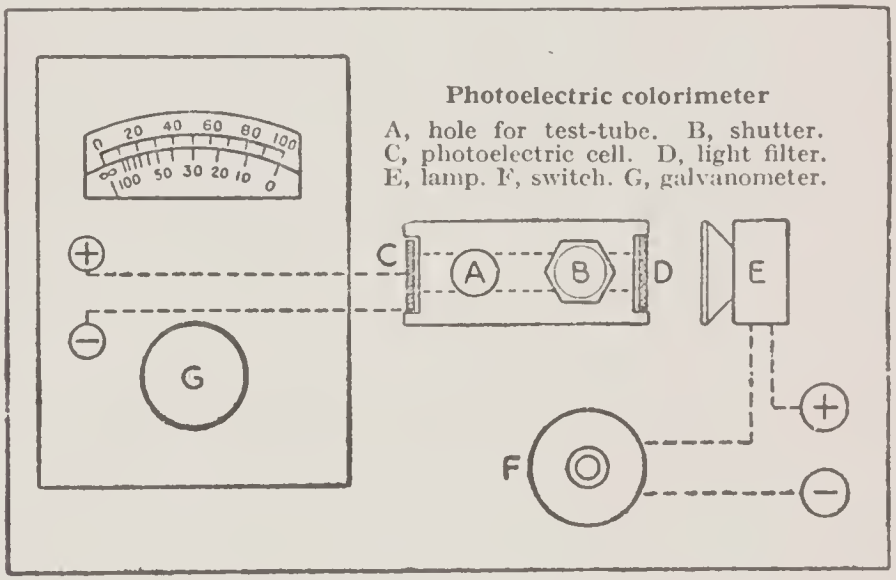


Fig. 21. Direct-reading Photoelectric Colorimeter.

which the light passes. In front of the glass cell or test tube is a light filter (D). A groove $\frac{7}{8}$ in. wide at the back of the block fits the photoelectric cell (C).

The photoelectric cell is of the selenium type, 22×40 mm. The 'EEL' electroselenium cell supplied by Messrs. Evans Electroselenium, of Harlow, Essex, has proved satisfactory. A copper wire, fitted on the surface of the rear groove in suitable position to make contact with the exposed strip of selenium which lies near the edges of the front surface of the cell, is connected to an electric terminal screwed into the back of the block. A lead from this front-surface terminal connects with

the negative pole of the galvanometer. A second terminal carries a spring clip which holds the photoelectric cell in place and makes contact with its back surface. The back-surface terminal is connected to the positive pole of the galvanometer.

Light filters of glass and gelatin have been used. The Chance red (OR 2), green (OGr 1), and blue-green (OB 2) glass filters have been found suitable for most purposes. Alternatively, the Ilford bright spectrum series of gelatin filters may be used (Fig. 22). The size is 1 × 2 in. Both the

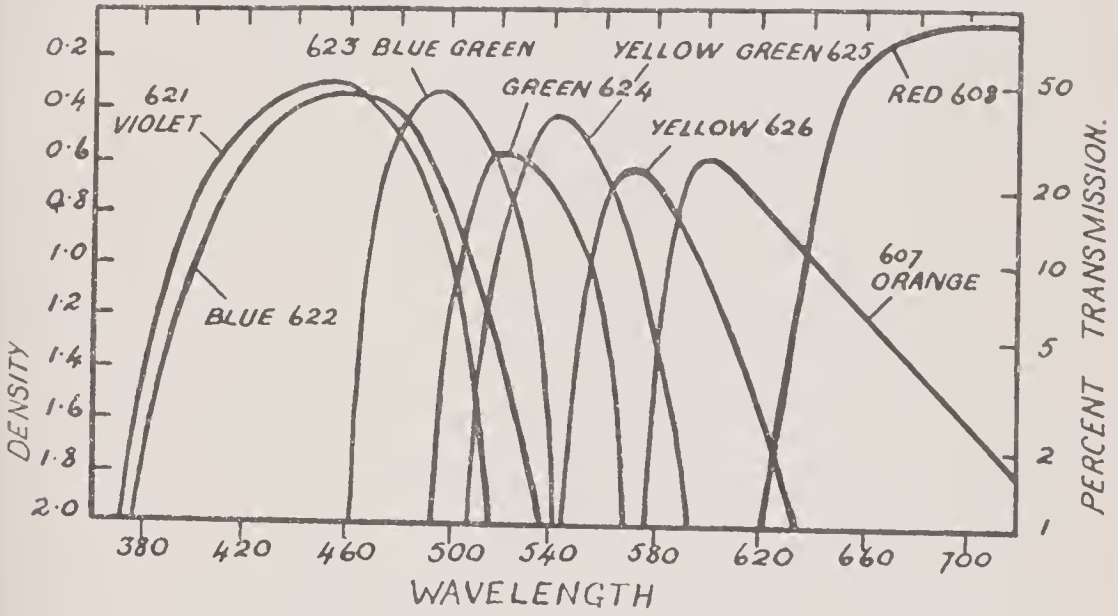


FIG. 22. Absorption curves of Ilford bright spectrum light filters.

sensitivity and accuracy of colour measurement are increased by the use of light filters. As in visual photometry, the measurement of colour intensity by photoelectric means is best carried out in light restricted to parts of the spectrum whose light is maximally absorbed by the coloured solution under investigation. This condition is approximated with the filters recommended.

The galvanometer for use with this instrument should be of 500–2000 ohms resistance and should have a maximum deflexion corresponding to 5–10 microamperes. The pointer type and the reflector type have been found equally satisfactory. The most convenient galvanometer scale is a

reversed logarithmic one on which the zero point corresponds to maximum deflexion. The reading is then directly proportional to the light absorbed. A set of coloured solutions containing varying amounts of the same pigment will give readings which are directly proportional to the concentration.

The logarithmic scale used is, in fact, that of the extinction 'E.' If the full deflexion of the galvanometer is divided into a linear scale of 100 equal parts, on which 0 corresponds to darkness and 100 to the point of full-scale deflexion with 100 per cent of incident light, then any readings less than 100 will correspond to percentages of light transmission which are less than 100 per cent of the incident light. They can be thought of as different degrees of extinction of the incident light. The extinction E or 'optical density' is defined as the logarithm of the ratio of incident to transmitted light—

$$\text{i.e. } E = \log (100/\% \text{ transmitted light}).$$

If two solutions of different optical density are interposed in the light path, then their extinctions E will be in the same relation one to the other as their concentrations of pigment —i.e. $E_1/E_2 = C_1/C_2$. This simple photometric expression of Beer's law defines the condition to be aimed at with a photoelectric colorimeter. To simplify the reading of E, and hence to simplify the calculation, the logarithmic scale is used on the galvanometer. It is arrived at in the following way. If, for instance, a coloured solution transmits only 50 per cent of the incident light—i.e. gives a reading of 50 on the linear scale—then its extinction $E = \log 100/50 = 0.301$. The point opposite 50 on the linear scale is marked 0.301 on the logarithmic scale. Similarly, 100 on the linear scale is 0 on the logarithmic, 10 on the linear is 1 ($\log 100/10 = 1$) on the logarithmic, and so on. For convenience, the values of E, which comprise the logarithmic scale, are multiplied by 100, and are plotted as whole numbers. The extinction E of a coloured solution is spoken of as the 'extinction coefficient' when it is determined at, or reduced to, a solution depth of unity—i.e. 1 cm.

OPERATION OF THE INSTRUMENT

The galvanometer is levelled, the suspension is released and the 'spot' or pointer adjusted to the ∞ mark of the logarithmic scale (0 on the linear scale) by turning the suspension knob. The light is now switched on. The shutter is turned up or down to regulate the amount of light reaching the photoelectric cell, until the needle is at 0 on the logarithmic scale. It should remain at this point and will do so after the first few minutes if the electric supply is taken from a properly charged accumulator or adequate transformer.

A set of test tubes (or glass cells) is selected which will fit the hole snugly but easily. They should be all of similar glass and free from obvious streaks and scratches. A tube (or cell) is filled with water, wiped with a towel to remove any stains and placed in the hole. The shutter is turned until the needle is at 0. A scratch is made on the side of the test tube opposite a mark on the block to indicate that the tube should always be used in that position. If on turning the tube in the hole more than a scarcely perceptible movement of the needle takes place the tube should be discarded since it contains flaws in the glass which are interfering with the passage of light. The other tubes are tested similarly and a mark made on each to indicate in which position it should be inserted to ensure equal light transmission. The test is now repeated with a coloured solution in place of water, and the tubes which give the same, or nearly the same, reading are selected. Those tubes which pass this test may now be used inter-changeably.

The appropriate light filter for a coloured solution is next tested for. This will usually be the filter which gives the highest reading—i.e. absorbs most light—and which gives most nearly a direct proportionality in reading with different concentrations of pigment. Generally speaking, its colour will be 'complementary' to that of the solution—for a blue or green solution a red filter, for a yellow solution a blue filter, and for a red solution a green filter. The selection of the filter is made as follows :—

With the filter and cell of water in place, the galvanometer needle is adjusted to 0 by turning the shutter. The cell of

water is removed and another containing the colour standard is inserted. The galvanometer needle is allowed to come to rest and the reading is recorded. On replacing the water the needle should return to 0. If it fails to do so, the light is again adjusted by turning the shutter and the reading for the standard is again taken. This operation is now repeated with a second standard of double the strength of the first. The reading of the second standard should be twice that of the first. If this condition is not fulfilled, another light filter is tried. If the proportionality is approximated but not reached, the fault probably lies in the presence of a 'blank' colour—i.e. a colour arising from the reagents which have been used to produce the colour by reaction with the standard substance. A 'blank' consisting of the reagents and water, with no added standard, is prepared and substituted for the water in the 0 adjustment. Proportionality should now be obtainable with the appropriate filter.

Use with Standards. The use of a natural standard—that is, a solution containing a known amount of the substance under investigation—is almost universal in colorimetric practice with Duboscq type instruments. The preparation of a standard each time a determination is made has another use besides furnishing a basis for colorimetric comparison—it is the easiest way of testing if anything has gone amiss in the analytical procedure. For this reason the use of standard solutions should be continued with the photoelectric colorimeter. With a logarithmic scale the calculation is simply that of direct proportionality—

Concentration of test = (Reading of test/Reading of standard) × Concentration of standard.

The factor Reading of test/Reading of standard is reversed from that used with the Duboscq instrument, since the proportionality is here a direct, whereas with the Duboscq it is an inverse proportionality. As in visual colorimetry the reading of the standard which is nearest the test should be used in the calculation.

Use without Standards. A coloured solution of given density will always absorb the same proportion of incident light,

provided the light is invariably of the same intensity and wave-length. With any given coloured solution and the proper light filter, therefore, the same difference in galvanometer deflexion between water and coloured solution should be obtained. This is the case for any one instrument; different instruments may differ sufficiently in details of construction to give slightly different deflexions with the same solution. By calibrating an instrument against a series of standards a factor may be derived or a graph constructed for the calculation of the concentrations of subsequent test solutions of unknown strength. But it must be recognized that the accuracy obtained in this way may not be as great as that which will result from the simultaneous comparison of a standard with the test solution. The reagents used in any procedure may vary slightly from day to day—e.g. by deterioration—and as a consequence the colour produced on one occasion may not be absolutely identical with that produced on another. Moreover, the selenium cell may suffer small alterations in its sensitivity and as a consequence the deflexion resulting from any given incident light may vary slightly. However, the accuracy obtainable may be sufficient for many routine examinations. If it is desired to use the colorimeter in this way, a separate set of standard graphs or factors should be established for each instrument.

TWO-CELLED PHOTOELECTRIC COLORIMETERS

The best known example of this type of instrument in this country is the Hilger Spekker absorptiometer. It consists of two photoelectric cells, carefully matched, which work in opposition. The amount of light absorbed by a coloured solution and falling on one photocell is compensated and measured by altering the amount of light, passing only through water, which falls on the other photocell. This is done by altering the size of a variable aperture until the intensity of light passing through it has been reduced by the same amount as is the case with the light passing through and partially absorbed by the coloured solution in front of the other photocell.

The two photocells are connected to a spot galvanometer. At the point of equal light intensities falling on the two photocells the electrical current contributed to the galvanometer by each of them is equal and opposite, and the reading is therefore at zero. Any alteration in the amount of light reaching one or other of the photocells, either by a change of the coloured solution in front of the one cell or an alteration in the size of the aperture before the other, will cause unequal electrical potentials, and the galvanometer needle or spot will move away from zero. Careful adjustment of the drum, which controls the constrictable aperture, will restore the balance. By this means the amount of filtered light absorbed by a coloured solution may be measured, and recorded in terms of the logarithmic scale on the drum.

The operation of the Spekker is described in full in the directions furnished with the instrument. Briefly, it is used as follows : light filters complementary to the colour of the test solution (see above) are placed in the slots on either side of the light, before the two photocells. The drum scale is set at zero, i.e. fully open, and the coloured test solution in an optical glass cell is placed on this side. The galvanometer is brought to zero by moving the adjustment of the diaphragm on the other side, where a glass cell of water is placed. Water, or 'blank' solution, is now substituted for the test, by moving the rack on which the glass cells are mounted. Less light is absorbed and the galvanometer spot moves away from zero. It is brought back by partially closing the variable aperture, when the drum reading will give a measure of the amount of light which was absorbed by the coloured test solution. A standard solution is read in the same manner.

The two-celled colorimeter may be used with standards or by means of graphic calibrations, as in the case of the one-celled instrument, and the calculation of results is similar.

FLAME PHOTOMETER

The flame photometer is a recently developed photoelectric colorimeter for determining the concentrations of metals in solution by measuring the intensities of the light emitted by

them when their solutions are sprayed into a gas flame, e.g. the yellow light produced when sodium ehloride is sprayed in solution into a Bunsen flame. If this yellow flame is allowed to illumine a photoeleetric eell, and the light from it is passed through a suitable eombination of light filters, so that nothing but the yellow light emerges, then the deflexion of a galvanometer attaehed to the photoeell is proportional, on a logarithmie seale, to the amount of sodium whieh is being added to the flame. In the same way potassium and other metals ean be determined, by isolating the light whieh they emit at certain defined wavelengths through suitable light filters. In this way it is possible to determine, e.g. sodium and potassium in the presenee one of the other, and of other salts of metals, by employing earefully selected combinations of light filters. A deseription of a simple flame photometer, whieh ean be easily made in any laboratory having a reasonably equipped workshop, has been given by Domingo and Klyne (1949); and a eommereial model is made by Evans Eleetroselenium Ltd., Harlow, Essex.

In biologieal work determinations of sodium and potassium are important, and many of these are required in the study of eertain diseases. The ehemieal proeedures for sodium and potassium are long and tedious, and the flame photometer forms a simple, aeeurate and speedy method of determining their eoncentration in urine, blood, plasma and C.S.F., with much less work than the ehemieal proeedures require.

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INTERNATIONAL ATOMIC WEIGHTS, 1947*

	Symbol	*Atomic weight*		*Symbol*	*Atomic weight*
Aluminium	Al	26.97	Molybdenum	Mo	99.95
Antimony	Sb	121.76	Neodymium	Nd	144.27
Argon	A	39.94	Neon	Ne	20.18
Arsenic	As	74.91	Nickel	Ni	58.69
Barium	Ba	137.36	Nitrogen	N	14.01
Beryllium	Be	9.02	Osmium	Os	190.2
Bismuth	Bi	209.00	Oxygen	O	16.00
Boron	B	10.82	Palladium	Pd	106.7
Bromine	Br	79.92	Phosphorus	P	30.98
Cadmium	Cd	112.41	Platinum	Pt	195.23
Calcium	Ca	40.08	Potassium	K	39.10
Carbon	C	12.01	Praseodymium	Pr	140.92
Cerium	Ce	140.13	Protactinium	Pa	231
Cesium	Cs	132.91	Radium	Ra	226.05
Chlorine	Cl	35.46	Radon	Rn	222
Chromium	Cr	52.01	Rhenium	Re	186.31
Cobalt	Co	58.94	Rhodium	Rh	102.91
Columbium	Cb	92.91	Rubidium	Rb	85.48
Copper	Cu	63.54	Ruthenium	Ru	101.7
Dysprosium	Dy	162.46	Samarium	Sm	150.43
Erbium	Er	167.2	Scandium	Sc	45.10
Europium	Eu	152.0	Selenium	Se	78.96
Fluorine	F	19.00	Silicon	Si	28.06
Gadolinium	Gd	156.9	Silver	Ag	107.88
Gallium	Ga	69.72	Sodium	Na	22.10
Germanium	Ge	72.60	Strontium	Sr	87.63
Gold	Au	197.2	Sulphur	S	32.07
Hafnium	Hf	178.6	Tantalum	Ta	180.88
Helium	He	4.00	Tellurium	Te	127.61
Holmium	Ho	164.94	Terbium	Tb	159.2
Hydrogen	H	1.01	Thallium	Tl	204.39
Indium	In	114.76	Thorium	Th	232.12
Iodine	I	126.92	Thulium	Tm	169.4
Iridium	Ir	193.1	Tin	Sn	118.70
Iron	Fe	55.85	Titanium	Ti	47.90
Krypton	Kr	83.7	Tungsten	W	183.92
Lanthanum	La	138.92	Uranium	U	238.07
Lead	Pb	207.21	Vanadium	V	50.95
Lithium	Li	6.94	Xenon	Xe	131.3
Lutecium	Lu	174.99	Ytterbium	Yb	173.04
Magnesium	Mg	24.32	Yttrium	Y	88.92
Manganese	Mn	54.93	Zinc	Zn	65.38
Mercury	Hg	200.61	Zirconium	Zr	91.22

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